THE ROLE OF $GABA_B$ RECEPTORS IN TEMPORAL LOBE EPILEPSY

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Abstract

Temporal lobe epilepsy is the most common partial epilepsy syndrome seen in adult humans. The hippocampus is a key structure in the evolution of temporal lobe seizures. The axons of the dentate granule cells, the mossy fibres, constitute a major hippocampal excitatory input. Inhibitory phenomena at mossy fibre synapses may therefore prevent seizure propagation through the hippocampus. One such inhibitory phenomenon is heterosynaptic depression. In this thesis I studied the role of $GABA_B$ receptors in temporal lobe epilepsy. In particular I studied changes in $GABA_B$ receptor-mediated heterosynaptic depression at the mossy fibre synapse following status epilepticus. I have shown that status epilepticus, triggered by either perforant path stimulation or pilocarpine administration, was followed 24 hours later by a loss of $GABA_B$ receptor-mediated heterosynaptic depression among populations of mossy fibres. This was accompanied by a decrease in the sensitivity of mossy fibre transmission to the exogenous $GABA_B$ receptor agonist baclofen. Autoradiography revealed a reduction in $GABA_B$ receptor binding in *stratum lucidum* after status epilepticus. I then addressed the question: what is the source of the GABA that mediates heterosynaptic depression? I have also shown that the $GABA_B$ receptor agonist baclofen has antiepileptic properties. In addition, $GABA_B$ receptors do not appear to be involved in the function of the antiepileptic drug tiagabine. Failure of $GABA_B$ receptor-mediated modulation of mossy fibre transmission may contribute to the development of spontaneous seizures after status epilepticus. The $GABA_B$ receptor may be useful as a target for antiepileptic drugs.

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Chapter 1: General Introduction

1.1 The study of epileptology

Throughout the ages people have been fascinated by the epileptic condition. The ancient Babylonians described epilepsy over three thousand years ago and the first known description is on a cuneiform tablet, which is housed at the British Museum, London. The Babylonians thought epilepsy had been sent by demons and sacrifices were offered to these demons in order to find a cure (Temkin, 1971). Different spirits were thought to cause different forms of seizures and detailed descriptions of these seizure types can be found in Babylonian texts. In 460 BC Hippocrates used the term epilepsy (from the Greek "epilambanein" meaning to seize or attack) to describe seizures and referred to the condition of recurrent seizures as the "sacred disease". Hippocrates believed that epilepsy did not come from spirits, demons or gods but was due to brain dysfunction. This perception was developed further in the 18th and 19th centuries. As neurology became a discipline distinct from psychiatry, the concept of epilepsy as a brain disorder became more widely accepted. During this time, the function of the brain was being studied and breakthroughs were being made that would shape modern neurology. In Berlin, around 1870, two German physicians, Gustav Theodor Fritsch and Eduard Hitzig performed experiments that involved stimulation of the cortex in awake dogs, and demonstrated that specific parts of the cortex are involved in movements of the contralateral limbs. They applied a galvanic current to the surface of the cerebral hemispheres and observed that stimulating specific cortical regions consistently caused movements in particular

muscles. Their work was continued by David Ferrier in London, who performed electrical stimulation of the cortex to map localization of function in such species as monkeys, dogs, jackals, cats and rodents, and ablation experiments in the brains of monkeys to confirm the results of his stimulation experiments (Ferrier, 1876).

The pathophysiology of epilepsy was first studied by one of the founders of modern neurology, John Hughlings Jackson. In 1873 he proposed a definition: "Epilepsy is the name for occasional, sudden, excessive, rapid and local discharges of grey matter". He also suggested that the character of the seizures depended on the location and function of the site of the discharges. He correlated ictal symptoms (the word ictus is used to refer to the actual fit itself) with the anatomical location of cerebral lesions that were identified on post-mortem examination. More recently, it is often stated that epilepsy arises due to an imbalance between excitation and inhibition, involving glutamate and γ amino-butyric acid (GABA) respectively (Tasker and Dudek, 1991). This view has emerged because blocking $GABA_A$ receptors induces epileptiform activity (Collins et al, 1983) and drugs that increase GABAergic inhibition stop seizures (Gale, 1992). However, this idea does not always hold true, as will be seen in this discussion.

1.2 Social Impact

Epilepsy is the most common acquired chronic neurological disorder. It has a world wide incidence of approximately 50-100 cases per 100,000 persons (higher in undeveloped countries) and a prevalence of between 4 and 10 per 1000 persons

(Sander, 1997). The life time risk of developing epilepsy is reported to be 2-5 % (Sander and Shorvon, 1996). Despite ongoing research and the emergence of new generation antiepileptic drugs, about 50% of patients treated with modern antiepileptic drugs continue to experience seizures (Pitkanen and Sutula, 2002), and about one third of epilepsy patients are refractory to treatment (Schmidt, 2002). Epileptic seizures may result in injury or death, especially tonic-clonic seizures (Kirby and Sadler, 1995). A diagnosis of epilepsy carries an excess mortality that is 2-3 times higher than that for the general population (Cockerell et al, 1994). Causes of this excess mortality include underlying disease such as stroke or brain tumour, suicide, accidents resulting from seizures, including drowning, status epilepticus and sudden unexpected death in epilepsy (SUDEP). SUDEP has been estimated to account for about 500 deaths each year in the UK (National Sentinel Clinical Audit of Epilepsy-Related Death, 2002). If epilepsy is intractable to medical treatment, then surgical management can be considered (Hirsch et al, 2000). In patients with temporal lobe epilepsy, this usually involves removal of the hippocampus and part of the anterior temporal lobe of the cerebral cortex (Spencer, 2002). Modern imaging techniques, cellular electrophysiological recording methods and molecular technologies are being applied to improve our understanding and treatment of epilepsy.

1.3 Modern definitions

John Hughling Jackson's definition of epilepsy has been developed into the modern definition of a seizure. A seizure is now defined as a clinical manifestation presumed

to result from an abnormal and excessive discharge of a set of neurons in the cortex (Hopkins et al, 1995). It is important to note that a seizure is a clinically observed event, which therefore means that an electrical event recorded *in vitro* is not a seizure. The characteristics of a particular seizure depend on the site of abnormal discharge within the brain. Seizures can either be either partial or generalised. A partial seizure occurs due to discharge of a localised group of cortical neurons whereas a generalised seizure occurs when generalised neuronal activity starts simultaneously in both hemispheres. These two categories are further subdivided into other seizure types. These are distinguished on clinical grounds and with the use of electroencephalography (EEG) (Table 1.1).

Table 1.1 Seizure classification

1. Partial (focal) seizures:
A. Simple – motor, somatosensory, autonomic, psychic
B. Complex
a. Impaired consciousness at outset
b. Simple partial followed by impaired consciousness
C. Partial (focal) seizures evolving to generalized tonic-clonic (GTC)
a. Simple to GTC
b. Complex to GTC
2. Generalized seizures (convulsive or non-convulsive) :
A. Absences
a. Absence seizures
b. Atypical absence seizures
B. Myoclonic
C. Clonic
D. Tonic
E. Tonic-clonic
F. Atonic
G. Combinations
3.Unclassified epileptic seizures

Consciousness is normal in simple partial seizures but impaired in complex partial seizures. The term "seizure" is not synonymous with "epilepsy". An individual may have an isolated seizure but this does not necessarily mean they have epilepsy. A commonly used modern definition of epilepsy is "the propensity to have seizures" and this encompasses many different epilepsy syndromes (Table 1.2).

Table 1.2 Epilepsy Classification

(Engel et al, 2004)

EPILEPSY SYNDROMES AND RELATED CONDITIONS
Benign familial neonatal seizures
Early myoclonic encephalopathy
Ohtahara syndrome
* Migrating partial seizures of infancy
West syndrome
Benign myoclonic epilepsy in infancy
Benign familial and non-familial infantile seizures
Dravet's syndrome
HH syndrome
* Myoclonic status in nonprogressive encephalopathies
Benign childhood epilepsy with centrotemporal spikes
Early onset benign childhood occipital epilepsy (Panayiotopoulos type)
Late onset childhood occipital epilepsy (Gastaut type)
Epilepsy with myoclonic absences
Epilepsy with myoclonic-astatic seizures
Lennox-Gastaut syndrome
Landau-Kleffner syndrome
Epilepsy with continuous spike-and-waves during slow-wave sleep (other than LKS)
Childhood absence epilepsy
Progressive myoclonus epilepsies
Idiopathic generalized epilepsies with variable phenotypes
Juvenile absence epilepsy
Juvenile myoclonic epilepsy
Epilepsy with generalized tonic-clonic seizures only
Reflex epilepsies
Idiopathic photosensitive occipital lobe epilepsy
Other visual sensitive epilepsies
Primary reading epilepsy
Startle epilepsy
Autosomal dominant nocturnal frontal lobe epilepsy
Familial temporal lobe epilepsies
*Generalized epilepsies with febrile seizures plus
*Familial focal epilepsy with variable foci
Symptomatic (or probably symptomatic) focal epilepsies
Limbic epilepsies
Mesial temporal lobe epilepsy with hippocampal sclerosis
Mesial temporal lobe epilepsy defined by specific etiologies
Other types defined by location and etiology

Neocortical epilepsies
    Rasmussen syndrome
    Other types defined by location and etiology
* Syndromes in development

CONDITIONS WITH EPILEPTIC SEIZURES THAT DO NOT REQUIRE A DIAGNOSIS OF EPILEPSY
Benign neonatal seizures
Febrile seizures
Reflex seizures
Alcohol withdrawal seizures
Drug or other chemically-induced seizures
Immediate and early post traumatic seizures
Single seizures or isolated clusters of seizures
Rarely repeated seizures (oligo-epilepsy)

Most seizures are self-limiting and last from seconds to a few minutes. A seizure event can be divided into four stages. The **prodrome** occurs days to hours before the seizure itself, and consists of altered behaviour or mood. The **aura** is a simple partial seizure that occurs prior to the major seizure and is a subjective or motor event perceived by the patient. Since an aura involves perception, this term is not usually used in animals, as it is not clear whether they experience aura in the same way humans do. The seizure itself is termed the **ictus**. There then follows a **post-ictal period** in which neurological abnormalities can occur. Occasionally seizure activity becomes prolonged; this is known as status epilepticus (SE). SE is defined as a single seizure of more than 30 minutes duration, or a series of epileptic seizures during which function is not regained between ictal events in a period lasting more than 30 minutes (Hopkins et al, 1995). This definition has recently been modified by

some workers for practical purposes to a period of seizure activity lasting five minutes or longer (Lowenstein et al, 1999).

1.4 Temporal lobe epilepsy

Temporal lobe epilepsy (TLE) is the most common epilepsy in adult humans. It is characterised by simple or complex partial and secondarily generalised seizures in various combinations. Febrile seizures and a familial history of epilepsy are often associated. The onset tends to be in childhood or young adulthood. The simple partial seizures tend to be autonomic, psychic or sensory. The most common form is an epigastric rising sensation. The complex partial seizures tend to follow the pattern of motor arrest, then oro-alimentary automatism, followed by other automatisms. There is an association between febrile seizures in infancy and temporal lobe epilepsy although the exact nature of this relationship is unclear. Many cases of febrile seizures appear to be benign, but prolonged complex febrile seizures are more consistently associated with subsequent temporal lobe epilepsy (Abou-Khalil et al, 1993). EEG may reveal asymmetry of background activity, temporal spikes, sharp and/or slow waves. Patients with intractable TLE often exhibit a pathological lesion known as hippocampal, or mesial temporal sclerosis, which will be discussed in more detail later. Recent experimental and human studies suggest that the development of epilepsy does not stop at the time of diagnosis, but that recurring seizures may contribute to the progression of the disorder. This progression may be associated with continuing molecular, cellular, or network changes.

1.5 Epileptogenesis

How does a normal brain develop the propensity to have seizures? Epileptogenesis is the process whereby structural and functional changes occur following a brain insult that result in epilepsy. Such insults include status epilepticus, febrile seizures, hypoxic-ischaemic injury and head trauma. In experimental models, insults such as chemoconvulsants and electrical stimuli are applied in order to stimulate epileptogenesis. These insults often result in an immediate selective neuronal loss, which is most prominent in areas CA1 and CA3 of the hippocampus. There then follows a latent period of variable length and it is presumed that structural and functional changes occur during this time which eventually lead to epilepsy. This latent period is also presumed to occur in humans after such insults as status epilepticus and head trauma. The latent period lasts from days to weeks following pilocarpine or kainic acid induced status epilepticus and can be several years in humans that have suffered head trauma (Herman, 2002). This latency suggests that either epileptogenesis is a long process, or that a second insult is necessary for epilepsy to occur. This "second hit hypothesis" suggests that an initial insult results in lowered seizure threshold, and then a later insult, the 'second hit' results in the expression of epilepsy (Walker et al, 2002c).

During the latent period, between insult and the expression of epilepsy, several functional and structural changes occur. Axons of the dentate granule cells (Proper et al, 2000), interneurons (Magloczky et al, 2000) and CA1 pyramidal neurons (Esclapez et al, 1997) undergo re-organisation and form functional aberrant circuits;

a process known as sprouting. Interneuronal populations are lost (Houser and Esclapez, 1996) and may receive fewer excitatory inputs (Doherty and Dingledine, 2001). $GABA_A$ receptors undergo altered subunit composition (Buhl et al, 1996; Kapur and Macdonald, 1997). Glutamatergic mechanisms also undergo changes including increased NMDA receptor activation (Lieberman and Mody, 1999). Research is currently underway to identify drugs that can be given in the latent period which will prevent the later development of epilepsy. This is important for the treatment of people that have suffered such insults as status epilepticus or head trauma so that development of epilepsy can be prevented.

1.6 Synchronous activity

Hypersynchronicity is often stated as being a part of the abnormal activity seen in the epileptic brain. Although increased synchronous activity is often linked with epileptiform activity, it was recently reported that before a seizure (during the preictal period) there is a decrease in synchronization, as measured by depth electrodes implanted into the hippocampi of epileptic patients (Mormann et al, 2003). From this study, decreased synchronization appears to be a consistent change which can be used to predict the onset of a seizure. During the seizure itself, neurons fire in a hypersynchronous manner. There have been several theories proposed as to how hypersynchronous firing occurs in the epileptic hippocampus. Intrinsic properties of CA3 pyramidal cells and high connectivity between cells allow generation of synchronized rhythmic bursts when $GABA_A$ receptors are blocked and it is proposed that this type of activity is analagous to interictal epileptiform activity

(Miles and Wong, 2003). It has recently been shown that $GABA_A$ receptor activation can be depolarising when the reversal potential for ions passing through $GABA_A$ receptors is more positive than resting potential (Stein and Nicoll, 2003). Depolarising GABAergic activity has been implicated in the synchronization of neuronal activity. Synchronous activity was recorded in the subiculum in slices of brain tissue from temporal lobe epilepsy patients which was similar to interictal activity recorded in scalp EEGs from the same patients. It was associated with depolarising potentials in subicular pyramidal cells which were blocked by $GABA_A$ receptor antagonists (Cohen et al, 2002). Non-synaptic forms of communication between neurons may also play a role in synchronising neurons, including electrical coupling through gap junctions, and ephaptic and field effects (Jefferys, 1995). Jefferys and Haas (1982) showed that oscillations occurred in CA1 in the absence of synaptic transmission. Draguhn et al (1998) showed that if they blocked chemical synaptic transmission in hippocampal slices by using a calcium free solution for their experiments, high frequency oscillations became more prominent although postsynaptic field potentials ceased. They then showed that these oscillations could be reversibly suppressed by octanol, halothane or carbenoxolone which have the common effect of blocking gap junctions. The oscillations were prolonged in the presence of NH_4Cl, which causes intracellular alkalinisation and enhances gap junction coupling. Synchronous *inhibitory* activity has also been implicated in epilepsy. Synchronous inhibition in the brain was first observed during normal physiological activity (Freund and Buzsaki, 1996). Several models of normal physiological rhythmic activity, including oscillations at theta (4-12Hz) (Cobb et al,

1995) and gamma frequencies (30-80 Hz) (Traub et al, 1999), depend on synchronous inhibitory activity. Bracci et al (1999) studied tetanically induced oscillations in CA1. They showed that the development of synchronized action potentials was induced by $GABA_A$ receptor mediated slow depolarisations and that the synchronization was caused by field effects. There is also burgeoning evidence that inhibitory synchronous activity is linked to epilepsy. Synchronous interneuronal bursting is proposed to cause high concentrations of GABA release and promote depolarizing responses (McBain, 1994). Velazquez and Carlen (1999) showed that epileptiform events in slices, produced by applying high frequency trains to Schaffer collaterals, recruit progressively more interneurons into a synchronized network. They suggest that GABAergic activity as well as glutamatergic activity is necessary for epileptiform activity in their model. Khazipov and Holmes (2003) recently demonstrated that kainate induced epileptiform activity *in vivo* in the gamma frequency band results primarily from synchronous inhibition provided by hippocampal interneurons. It seems then that the view of epilepsy as reduced inhibition or increased excitation is overly simplistic.

1.7 The hippocampus

The hippocampus is a key structure in temporal lobe epilepsy. Pathological changes are observed in the hippocampus in some patients with drug resistant TLE. Its anatomy and relation to other local structures is complex. It is part of a region of the brain known as the limbic system. In 1878 Broca observed that the mammalian brain has a horseshoe shaped rim of grey matter that surrounds the junction between the

diencephalon and each cerebral hemisphere. He referred to this rim of cortex as the limbic lobe (from the Latin *limbus* meaning "border"). This tissue is now referred to as the limbic system although there is some debate as to its boundaries. Table 1.3 lists the structures that most authors include within the limbic system. Some authors suggest that certain regions of the thalamus, fornix and the mammillary bodies are also included.

Table 1.3 Key structures of the limbic system

Cingulate and parahippocampal gyri
Hippocampus
Amygdala
Septal nuclei
Hypothalamus
Parts of midbrain reticular formation
Olfactory areas

The hippocampal formation consists of six cytoarchitectonically distinct regions: dentate gyrus, hippocampus (or hippocampus proper), subiculum, presubiculum, parasubiculum and entorhinal cortex. During development the medial edge of the temporal lobe expands so that the hippocampus ends up occupying the floor of the temporal horn of the lateral ventricle. The hippocampus itself is a curved and recurved sheet of cortex folded into the medial surface of the temporal lobe. The subiculum is a transitional zone continuous with the hippocampus proper at one of

its edges and the cortex of the parahippocampal gyrus at the other edge. The alveus is a thin layer of white matter which separates the hippocampus from the lateral ventricle. It consists of fibres that arise from the pyramidal cells of the hippocampus and that are directed towards subcortical termination sites or to the contralateral hippocampal formation. The hippocampus proper is also known as the Cornu Ammonis (CA) or literally the horn of Ammon, after an Egyptian deity with the head of a ram. It can be divided into 4 fields (CA1-4). The palisade of cell somata starts from the end of the subiculum and continues from CA1 through to CA4 which is positioned between the blades of the dentate gyrus. The cerebral cortex is generally a six-layered structure. However, the six-layered entorhinal cortex becomes four-layered at the subiculum and then three-layered at the Cornu Ammonis. The hippocampus proper consists of 1) molecular, 2) pyramidal and 3) polymorphic layers. This can be further divided into 5 strata. These are: stratum oriens, stratum pyramidalis, stratum radiatum, stratum lacunosum and stratum moleculare.

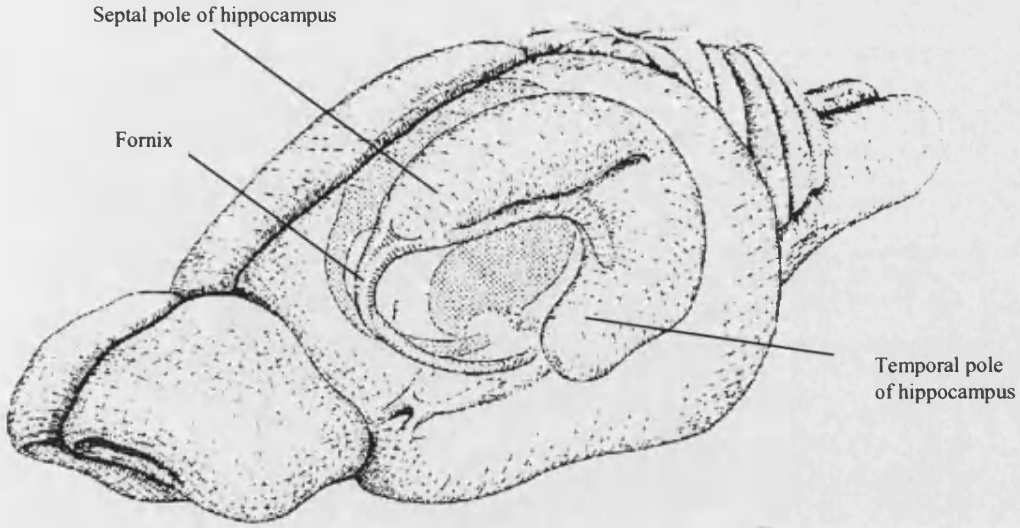


Figure 1.1: The hippocampus of the rat in situ (from Amaral and Witter, The Rat Nervous System, 1995, Academic Press).

Table 1.4 Components of the strata of the hippocampus

Stratum	Cell types and structures
S. oriens:	axons of pyramidal neurons, somata and dendrites of basket cells, interneurons
S. pyramidalis:	pyramidal cell somata, interneurons
S. radiatum:	apical dendrites of pyramidal neurons, CA3-CA3 associational connections, CA3-CA1 Schaffer collateral connections, interneurons
S.lacunosum + S. moleculare:	perforant path axons from entorhinal cortex, interneurons

Area CA3 has an additional layer between s.pyramidale and s.radiatum, called *stratum lucidum* which contains the axons of the dentate granule cells

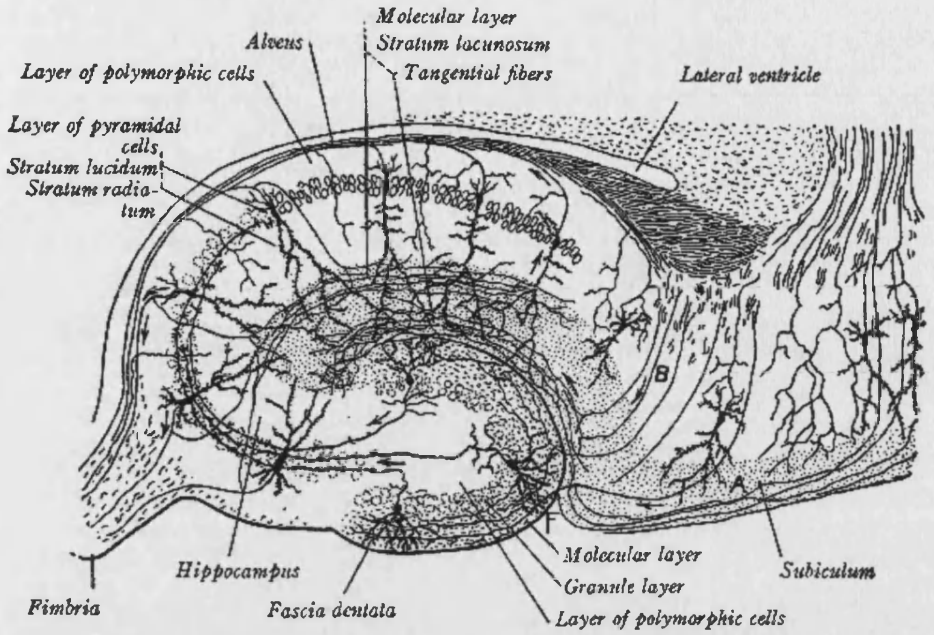


Fig. 235.—Diagram of the structure and connections of the hippocampus. The arrows show the direction of conduction; *A*, molecular layer, and *B*, pyramidal cell layer of the subiculum; *F*, hippocampal fissure. (Cajal.)

Figure 1.2: Diagram showing the connections of the hippocampus (Cajal)

(Blackstad et al, 1970), which are termed mossy fibres. Interneurons are scattered throughout all of these layers. The dentate gyrus is also a three-layered structure consisting of: molecular layer, granule cell layer and polymorphic cell layer.

1.8 Hippocampal sclerosis

Bouchet and Cazauvieilh were the first to make an association between pathological changes in the hippocampus and epilepsy in 1825 (Temkin, 1971) during their work on potential links between mental disorders and epilepsy. During these studies they observed pathological changes in the hippocampus in five epileptic patients. The hippocampi had become firm in texture. They termed this pathological change "sclerosis". Then in 1880, Sommer reviewed the literature on the role of the

hippocampus in epilepsy. He was probably the first person to associate the hippocampus with temporal lobe seizures. He postulated that hippocampal cells were vulnerable to insults and that the resulting Ammon's horn sclerosis was the cause of epilepsy (Fisher et al, 1998). In 1899 Bratz described the microscopic appearance of hippocampal sclerosis (HS):-

1. Destruction of pyramidal neurons in Ammon's Horn (hippocampus proper), particularly in CA1
2. Loss of hilar neurons and CA4 pyramidal neurons (endfolium sclerosis)
3. Granule cell loss

He also showed that epilepsy is not always associated with hippocampal sclerosis. He thought that hippocampal sclerosis caused certain types of seizures rather than being a result of seizures. Certainly there is evidence that hippocampal sclerosis could indeed cause seizures. Most patients with refractory mesial TLE have hippocampal sclerosis (Babb and Brown, 1987). There is usually cell loss in both hippocampi although it tends to be asymmetrical. Unilateral hippocampal atrophy is often seen in these patients which is characterised on MRI by reduced volume and increased signal on T2 scans. The location of the seizure focus frequently corresponds to the side of the atrophic hippocampus (Fish and Spencer, 1995). Removal of the sclerotic tissue during temporal lobe resection successfully "cures" 80 % of patients (Arruda et al, 1996). However, among patients with hippocampal

atrophy, around 10% have bilateral atrophy. In these patients surgical success is achieved not by removing the more or less atrophied hippocampus, but by removing the seizure focus as identified by EEG recordings (Fish and Spencer, 1995). Later in the twentieth century an alternative explanation developed. A study by Scheibel et al (1974) demonstrated a positive correlation between the chronicity of the seizure disorder and the severity of the hippocampal damage which led many to believe that hippocampal sclerosis was an eventual consequence of chronic seizures rather than a cause. From the 1950s onwards, workers started to associate hippocampal sclerosis with prior cerebral injury early in life. Retrospective studies frequently show that hippocampal sclerosis is associated with prolonged febrile seizures in childhood (Lewis, 1999; Davies et al 1996), but see Tarkka et al (2003). However hippocampal sclerosis sometimes is present in the absence of any apparent initial precipitating injury. Despite the link between febrile seizures and TLE, many children who have had febrile seizures do not develop hippocampal sclerosis or TLE (Tarkka et al, 2003). It can be seen then that there is a complex relationship between early cerebral injury, temporal lobe epilepsy and hippocampal sclerosis.

The question then arises: how does hippocampal sclerosis contribute to epileptogenesis? Re-organisation of mossy fibre axons ("sprouting") occurs in both animal models and human patients with TLE into the inner molecular layer of the dentate gyrus. The role of these aberrant fibres has been a subject of controversy. The two lines of thought are either that sprouted fibres synapse onto dentate granule cells and form recurrent excitatory synapses, or that they synapse mainly onto

interneurons, thus increasing inhibition in the epileptic hippocampus. There are several lines of evidence showing that sprouted fibres make aberrant connections with granule cells both in animal models of epilepsy (Okazaki et al, 1995; Buckmaster et al, 2002) and human patients (Zhang and Hauser, 1999), resulting in functional recurrent excitatory synapses (Tauck and Nadler, 1985). More recently, Buckmaster et al (2002) provided evidence that most new synapses made by sprouted mossy fibres were with GABA-negative dendritic spines. In contrast to these findings Sloviter (1992) suggested that mossy fibre sprouting restores inhibition by innervation of interneurons by the sprouts and electron microscopic studies have revealed that sprouted mossy fibres probably synapse onto interneurons as well (Kotti et al, 1997). What are the functional consequences of sprouting and is it necessary for epileptogenesis to occur? Longo and Mello (1997) showed that if sprouting is inhibited by cycloheximide (a protein synthesis inhibitor) in laboratory models of epilepsy, epileptogenesis still occurs. In contrast with this work however, Williams et al (2002) failed to prevent sprouting with cycloheximide although they did show that spontaneous seizures could develop in the absence of Timm-stain positive mossy fibre sprouting. Similarly, spontaneous recurrent seizures develop even in the absence of sprouting in a modified kainic acid model of epilepsy (Zhang and Hauser, 1999). But, in this last study, the presence of sprouting and neuronal cell loss was associated with more severe seizures. These results do not necessarily mean that mossy fibre sprouting is not important in increasing hippocampal excitability; they just demonstrate that sprouting is not required for recurrent seizures to develop.

1.9 Animal Models

There are animal models of epileptic activity in such diverse species as *Drosophila* and non-human primates. These can be separated into models of seizures and models of epilepsy, although some models can be considered to be both. For example if flurothyl is administered to rodents they have a generalised seizure but do not develop spontaneous seizures. In contrast, if status epilepticus is induced by administration of pilocarpine the animal develops spontaneous seizures after a latent period of days to weeks. Thus the former case is a model of seizure activity but not of epilepsy, and the latter is a model of an acutely precipitated seizure leading to a chronic epileptic state. Some studies refer to models as "acute" e.g. maximal electroshock, pentylenetetrazol – models that produce immediate seizure activity – and "chronic" models that involve an insult such as status epilepticus which is followed by spontaneous seizures following a latent period. Also, study of the latent period may reveal changes that are relevant to epileptogenesis. There are also many species in which epilepsy occurs spontaneously, often due to a known or suspected genetic predisposition e.g. dogs, rats, mice, hamsters.

Transgenic mice with seizure disorders have also been developed (Upton and Stratton, 2003). These become important when considering that at least 40-50% of all forms of human epilepsy are idiopathic generalised epilepsies, which are characterised by lack of antecedent disease and have a presumed genetic origin. Transgenic models include those engineered with functionally identical mutations to those in human inherited epilepsy, models which have single altered genes to

determine whether those genes are involved in epileptogenesis and lastly, spontaneous mutations in mice, which have provided a source of potential candidate genes.

There are several different ways in which recurrent seizures can be induced in a normal animal (Fisher, 1989). These include administration of chemoconvulsants (either locally or systemically) and electrical stimulation of the brain. Chemoconvulsants that are used to produce animal models of epilepsy include pilocarpine, kainic acid, pentylenetetrazol and penicillin. Electrical protocols include maximal electroshock, amygdala kindling, and perforant path stimulation. Epileptiform activity can also be induced in a brain slice *in vitro*. This can be achieved by applying drugs, electrical stimuli, manipulating ion concentrations, or combinations of these. Such drugs include 4-aminopyridine, pilocarpine and the $GABA_A$ receptor antagonist bicuculline. Raising the concentration of potassium or lowering the concentration of magnesium can also induce epileptiform activity. The activity in these *in vitro* models can then be measured by making extracellular field potential recordings in the desired region or using multiple electrode arrangements. Propagation of epileptiform activity through a slice can also be monitored by measuring intrinsic optical signal (IOS). IOSs are generated *in vitro* by changes in light scattering properties through refraction and reflection and/or by changes in light absorption. These phenomena are mainly secondary to cell swelling leading to alterations in the extracellular space volume (D'Arcangelo et al, 2001). The relevance of synchronised bursts in the limited network of the slice preparation to

the *in vivo* situation is uncertain, especially since certain synchronised bursts *in vivo* have been proposed to be very different from seizures and even to have an antiepileptic effect (de Curtis and Avanzini, 2001). In this work I used both a chemical (systemic pilocarpine) and electrical (stimulation of the perforant path) stimulus to induce status epilepticus in rats.

Table 1.5 Commonly used models of epilepsy, seizures, and epileptiform activity

A) In vivo	**i) Chemical**	Pilocarpine (+/- lithium)
		Kainic acid
		Tetanus toxin
		Pentylenetetrazol
		Fluorothyl
		Penicillin
	ii) Electrical	Kindling
		Perforant path stimulation
		Maximal Electroshock
	iii) Genetic	Spontaneous
		Transgenic
B) In vitro (slice preparations)		4-aminopyridine
		Low magnesium
		Pilocarpine

1.9.1 Pilocarpine model

Turski et al (1983) were the first to demonstrate that systemic administration of pilocarpine, an agonist of muscarinic acetylcholine receptors, induced limbic seizures in rats. The seizures consisted of staring spells, olfactory and gustatory automatisms and motor limbic seizures that developed over 1-2 hours and built up progressively into limbic status epilepticus. The earliest electrographic alterations that they observed were in the hippocampus; these then propagated to the amygdala and cortex. A pattern of cell loss occurred in the hippocampus that is similar to human patients with TLE. The pilocarpine model can be used to ask different questions depending on the time period studied. We can study a) the immediate consequences of SE, b) the changes that occur during epileptogenesis (i.e. the period between SE and the onset of spontaneous seizures) or c) the period of spontaneous recurrent seizures.

1.9.2 Perforant path stimulation

Sloviter and Damiano (1981) developed a model of seizure induced neuronal damage which involved continuous electrical stimulation of the perforant path of urethane anaesthetised rats for twenty-four hours. The electrophysiological and pathological findings were similar to other models such as the kainic acid model. The stimulation evoked granule cell population spikes (due to the summation of action potentials from many neurons), epileptiform discharges and reduced dentate inhibition, which could be quantified by measuring loss of paired pulse inhibition. Hilar interneurons and CA3 pyramidal neurons were damaged. McIntyre et al (1982)

then modified the model by stimulating the perforant path in *unanaesthetised previously kindled* rats for sixty minutes at a frequency of 60 Hz. Since the process of kindling itself induces epilepsy, models were then developed in which the perforant path was stimulated in naïve rats and self-sustaining status epilepticus was eventually induced (Mazarati et al, 1998). Several different stimulating protocols have been used, including either intermittent or continuous stimulation. The stimulation protocol that I used had been modified by Walker et al (1999) from previous protocols described by Sloviter and Damiano (1981) and Rogers et al (1989). This consists of continuous stimulation of the perforant path at 20 Hz for two hours via electrodes implanted into the angular bundle. Activity is recorded in the dentate gyrus via another electrode throughout the procedure. In this model, electrographic and behavioural seizure activity becomes self-sustaining and persists after the stimulation has stopped.

These limbic seizures can be graded according to their severity using Racine's classification of kindled seizures (Table 1.6).

Table 1.6 : Development of kindled seizures after Racine (1972).

Stage	Seizure type
1	Mouth and facial movements
2	Head nodding
3	Forelimb clonus
4	Rearing
5	Rearing and falling

1.10 The mossy fibre – CA3 synapse

There are approximately 15 million dentate granule cells in the human dentate gyrus and about one million in the rat. They are unusual neurons, in that new dentate granule cells are formed throughout life, which are generated from stem cells located in the hilus (Palmer et al, 2000) and then migrate outwards into the granule cell layer (Kaplan and Bell, 1984). Despite the development of new cells throughout life, the number of granule cells does not apparently increase as the animal gets older (McEwen, 1999). Each cell gives rise to a single unmyelinated mossy fibre axon which leaves the dentate gyrus and forms fine collaterals that provide input to inhibitory interneurons and excitatory mossy cells within the dentate hilus. The main axons synapse onto the proximal apical dendrites of the CA3 pyramidal cells in *stratum lucidum* (Henze et al, 2000). The mossy fibres travel transversely through

the hippocampus in a lamellar arrangement (Andersen et al, 1969). The propagation of limbic seizures through the hippocampus has been demonstrated by recording activity *in vivo* in animal models (Collins et al, 1983). The hippocampal circuitry is often described in a simplified manner as a trisynaptic excitatory loop. Neurons from the entorhinal cortex project via the perforant path to granule cells. They project their axons, the mossy fibres, to CA3. Schaffer collaterals then project to CA1 and CA1 neurons project back to the entorhinal cortex via the subiculum.

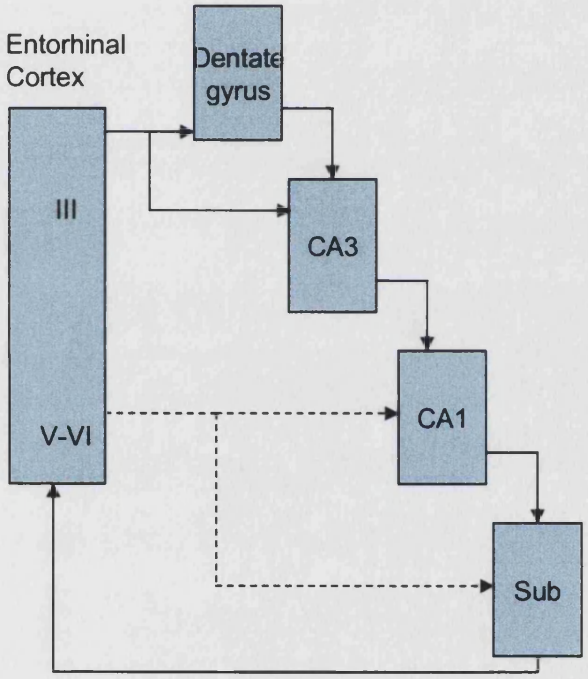


Figure 1.3: the "trisynaptic" circuit of the hippocampus. Sub = subiculum. Roman numerals refer to layers of entorhinal cortex.

Mossy fibres also synapse with other cell types in the hippocampus. Each mossy fibre makes approximately 11-18 synapses with CA3 pyramidal cells, 140-150 with cells of the hilus (which are mainly inhibitory) and 40-50 synapses with inhibitory interneurons within *stratum lucidum* (Acsady et al, 1998; Claiborne et al, 1986).

The dentate gyrus is often considered to be a gate or barrier to seizure activity propagating towards the hippocampus (Collins et al, 1983). However, the hippocampal input is also regulated at the mossy fibre - CA3 synapse and inhibitory phenomena occuring at this synapse will gate seizure activity into the hippocampus proper. Presynaptic group II metabotropic glutamate receptors (Kamiya et al, 1996) and $GABA_B$ receptors (Min et al, 1998; Vogt and Nicoll, 1999) are present on mossy fibre terminals and depress transmitter release from mossy fibres when activated. The regulation of inhibitory gating at this synapse and the mechanisms underlying changes in this gating during the development of epilepsy are the main subjects of this thesis.

1.10.1 Mossy fibre pharmacology

Mossy fibres release glutamate which targets AMPA and small numbers of NMDA receptors (Jonas et al, 1993), kainate receptors (Castillo et al, 1997), and group I metabotropic glutamate receptors (Kapur et al, 2001) on CA3 pyramidal cells. They also contain high concentrations of vesicular zinc (Frederickson et al, 2000) which is released from mossy fibres in a frequency dependent manner (Li et al, 2001). The role of mossy fibre zinc is not fully understood. Although Lu et al (2000) and Li et al (2001) argued that it plays a critical role in the induction of mossy fibre long-term potentiation (LTP), Vogt et al (2000) reported that mossy fibre LTP was intact in the *mocha* mouse in which the ZnT3 transporter which is responsible for packaging of zinc into vesicles is absent. The role of zinc in mossy fibre LTP thus remains

unclear. Mossy fibre zinc also modulates $GABA_A$ receptors (Ruiz et al, 2004) and NMDA receptors (Molnar and Nadler, 2001). Mossy fibres also release ATP/adenosine which plays an automodulatory role via presynaptic A1 receptors (Yamamoto et al, 1993). Tonic inhibition at presynaptic A1 receptors contributes to the release properties at this synapse (Moore et al, 2003). They also contain several peptides including dynorphin (Terman et al, 2000), enkephalin (Racz et al, 1998), cholecystokinin (Chandy et al, 1995), neuropeptide Y (Marksteiner et al, 1990) and neurokinin-B (Schwarzer et al, 1995). Dynorphin is released during high frequency stimulation and causes a heterosynaptic depression of neighbouring mossy fibres via kappa receptors on mossy fibre terminals (Weisskopf et al, 1993). Brain-Derived Neurotrophic Factor (BDNF) is also expressed at high concentrations in mossy fibres (Conner et al, 1997), and may serve a regulatory function in network excitability. Finally, although the mossy fibre-CA3 synapse has traditionally been considered to be an excitatory synapse, mossy fibres also contain the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) and GABA itself in both rodents (Sloviter et al, 1996) and primates (Sandler and Smith, 1991). They contain mRNA for the vesicular transporter for GABA, VGAT, which is age dependent (Gutierrez et al, 2003) and increases after synaptic stimulation of mossy fibres in slices (Lamas et al, 2001). Bergersen et al (2003) have recently shown that glutamate and GABA are co-localised in mossy fibre terminals and that the GABA is spatially correlated with synaptic vesicles. The anatomical demonstration of the co-localisation of GABA and glutamate is not evidence that the synapse necessarily releases both transmitters. Recently however, Walker et al (2001) showed that

$GABA_A$ receptor mediated IPSCs with characteristics typical of mossy fibre responses can be recorded in CA3 pyramidal neurons when stimuli designed to recruit mossy fibres are applied to acute hippocampal slices from healthy guinea-pigs. Also, Gutierrez and Heinemann (2001) recorded a GABAergic signal in CA3 pyramidal cells using a stimulus designed to selectively recruit mossy fibres in kindled rats that was not present in controls. The physiological role of GABA release from mossy fibres is not known. Since the GABA concentration in mossy fibres is increased after seizures, it could lead to a compensatory increase in inhibition after sustained epileptic activity. There is some evidence that GABA is only released at mossy fibres in juvenile control animals but not in adults (Gutierrez et al, 2003) suggesting that mossy fibre GABA could also have a role in development (Walker et al, 2002b; Kasyanov et al, 2004).

1.10.2 Mossy fibre physiology

Mossy fibre synapses are unusual in that they show marked use-dependent facilitation even at low frequencies (Salin et al, 1996); thus on increasing the stimulation frequency from 0.05 Hz to 0.2 Hz, there is a 2-fold increase in the amplitude of the field EPSP. They have presynaptic $GABA_B$ receptors (Hirata et al, 1992) that inhibit voltage gated Ca^{++} channels when activated and so reduce transmitter release. Presynaptic group II metabotropic glutamate receptors also suppress transmitter release at mossy fibre synapses (Kamiya et al, 1996). The activation of postsynaptic group I metabotropic glutamate receptors on the CA3 pyramidal neurons induces release of Ca^{++} from internal stores (Kapur et al, 2001).

The mossy fibre synapse is also influenced by inhibitory activity at several different levels. Since each mossy fibre synapses with more inhibitory cells than excitatory cells (Acsady et al, 1998) the inhibitory effects of mossy fibres on the hippocampal circuitry may be substantial. However, the extent to which this alters the strength of mossy fibre input to CA3 pyramidal neurons is not fully understood because of the complexity of the numerous interneuron types that are activated by hippocampal input (Urban et al, 2001). Also, mossy fibres synapse with CA3 pyramidal cell dendrites at a proximal position and so firing of CA3 pyramidal neurons is not likely to be strongly influenced by GABAergic synapses that are positioned more distally. It is likely then that presynaptic receptors on the mossy fibre synapse that depress transmitter release have a more dominant role in limiting mossy fibre transmission than feedforward inhibition from the dentate gyrus. Vogt and Nicoll (1999) and Min et al (1998) observed that a high frequency train of impulses applied to one mossy fibre pathway depresses transmission in an adjacent mossy fibre pathway. This phenomenon was termed heterosynaptic depression (HD). In the guinea-pig hippocampus this is mediated by $GABA_B$ receptors and metabotropic glutamate receptors. If heterosynaptic depression was altered after status epilepticus this could alter hippocampal excitability. I therefore asked the questions:

Is heterosynaptic depression present in the adult rat hippocampal mossy fibre synapse?

Is it altered after SE ?

1.10.3 Long-term potentation (LTP) at the mossy fibre synapse

LTP is a synaptic phenomenon that is considered to be currently the best experimental model for the synaptic basis of learning and memory. It is a long-term increase in synaptic strength that can occur subsequent to repetitive high frequency activity or paired pre- and post-synaptic activity. LTP that is dependent on simultaneous (within 100-150 ms) pre- and post-synaptic activity is termed "Hebbian" (or "associative") after a learning rule proposed by Hebb in 1949. The Hebb rule states that synapses increase in strength when there is conjunctive presynaptic and postsynaptic activity. Two distinct types of LTP are recognised on the basis of their requirement for the synaptic activation of N-methyl-D-aspartate (NMDA) receptors. Most forms of LTP, such as that at the Schaffer collateral synapse in CA1, are NMDA receptor dependent. It is the unique properties of the NMDA receptor that provide a Hebbian nature to these forms of LTP. At resting membrane potentials, NMDA receptors are blocked by magnesium. The block is only released when the membrane is depolarised by activation of other receptors such as AMPA receptors. At the mossy fibre-CA3 synapse however, LTP is independent of NMDA receptor activation (Harris and Cotman, 1986). Interestingly, depending on the induction protocol, mossy fibre LTP can be Hebbian or non-Hebbian. Long-lasting high frequency stimulation induces LTP regardless of the level of postsynaptic hyperpolarisation (Langdon et al, 1995) whereas brief stimulation induces LTP that also requires depolarisation of the postsynaptic cell. This form requires calcium influx via post-synaptic voltage dependent L type calcium channels (Kapur et al, 1998) thus requiring postsynaptic depolorization

which activates the channels. If induced by long-lasting stimulation however, calcium release from postsynaptic intracellular stores is sufficient to induce LTP (Yeckel et al, 1999).

More recently the role of the kainate receptor in presynaptic calcium entry has been recognised. Kainate receptors have now been shown to be involved in both the induction of mossy fibre LTP and in synaptic facilitation of mossy fibre synaptic transmission (Bortolotto et al, 1999; Contractor et al, 2001; Lauri et al, 2001 ; Schmitz et al, 2001). However the subtype of kainate receptor responsible for these properties has been a subject of controversy. The selective GLU_{K5} (previously known as GluR5) antagonist LY382884 blocks both processes (Lauri et al, 2001) but broad spectrum glutamate receptor antagonists, such as kynurenic acid and CNQX, which are active at GLU_{K5} receptors have been reported not to block the induction of mossy fibre LTP (Weisskopf and Nicoll, 1995; Yeckel et al, 1999). In fact, gene knockout studies support a role for GLU_{K6} (GluR6) but not GLU_{K5} receptors in mossy fibre LTP and synaptic facilitation (Contractor et al, 2001). Recent work has resolved some of the controversy. Lauri et al (2003) report that calcium entry through kainate receptors leads to calcium release from intracellular stores, which plays a role in both synaptic facilitation and the induction of LTP. I_h channels have also been implicated (Mellor et al, 2002) although this is controversial (Chevaleyre and Castillo, 2002).

1.12 The CA3 network

What is the role of the CA3 region of the hippocampus? It has been proposed that CA3 acts as an autoassociative network. It is ideally suited for this task because of its abundant excitatory connections among pyramidal neurons. The term "autoassociative" refers to a system of memory storage. In this type of system, memories are stored as a pattern of neural activity that can be recalled when a fragment of the memory is presented to the network. The recall of this memory is known as "completion". Such a network can learn each memory in one trial. The architecture of such a memory system has been proposed by Rolls and Treves, (2003). It has been hypothesized that memories are initially stored in the hippocampus and then transferred to the neocortex as more experience is gained and more generalised memories can be stored (McClelland and Goddard, 1996).

1.13 Altered inhibition in epilepsy

Blocking GABAergic inhibition produces seizures. Therefore a loss of GABAergic inhibition could be a key event in epileptogenesis. Loss of inhibition is frequently observed in epilepsy models and tissue from patients. Paradoxically though, other studies have reported increased inhibition in epilepsy. Sloviter proposed the "dormant basket cell hypothesis" to account for data obtained in rats that had been exposed to prolonged perforant path stimulation under urethane anaesthesia (Sloviter, 1983; Sloviter, 1987; Sloviter, 1991b). He observed that principal cell disinhibition and hyperexcitability immediately followed prolonged seizure discharges, correlated closely with selective neuronal injury to hilar neurons and

CA3 pyramidal cells, and were similar to those changes produced acutely by bicuculline (Sloviter, 1991b). He suggested that principal cell disinhibition might be caused by the loss of mossy cell activation of dentate basket cells and the loss of CA3 pyramidal cell excitation of area CA1 basket cells (Sloviter, 1991b). Findings by Bekenstein and Lothman (1993) supported the hypothesis as have several subsequent studies (Mangan et al, 1995; Jefferys and Traub, 1998; Morin et al, 1999; Doherty and Dingledine, 2001; Denslow et al, 2001). In contrast, Esclapez et al (1997) argued that in CA1, inhibition remained intact. Sloviter later suggested that if inhibitory interneurons do indeed survive, perhaps a weak glutamate receptor agonist might restore inhibition by selectively activating surviving inhibitory interneurons (Sloviter, 1991b) This has subsequently been supported by Khalilov et al (2002) in which a GluR5 subunit-containing glutamate receptor agonist selectively excites hippocampal interneurons and evokes hippocampal principal cell inhibition. Despite a lot of evidence that there is decreased inhibition in the epileptic hippocampus, there have been several conflicting findings. In the dentate gyrus, both decreased and increased inhibition has been observed. An increase in paired-pulse inhibition of granule cells has been observed in several epilepsy models. This was observed in the amygdala kindling model (Tuff et al, 1983) and in the kainic acid model (Haas et al, 1996; Buckmaster and Dudek, 1997). Granule cells from epileptic rats have increased $GABA_A$ receptor current density when compared with controls (Gibbs et al, 1997) and there are more $GABA_A$ receptors per synapse in kindled animals when compared with controls (Otis et al, 1994; Nusser et al, 1998). There are a number of explanations for these contrasting findings. First, studies are often

not directly comparable because they use different animal models. Second, inhibition is measured in different ways including: paired pulse inhibition, miniature inhibitory post-synaptic current (mIPSC) and spontaneous inhibitory post-synaptic current (IPSC) frequency, and evoked inhibitory post-synaptic potential (IPSP). Third, it has been proposed that results may differ depending on the septotemporal level of the hippocampus examined (Bernard et al, 2000). *In vivo* studies in rats tend to concentrate on the more accessible septal level of the hippocampus, however tissue resected from humans includes the anterior (the human equivalent to the rat temporal) region (NB in the human the hippocampal poles are termed anterior and posterior, in contrast to the rat that is described as having temporal and septal poles). It is here that hilar neuron loss is most severe (Babb et al, 1984) and where the number of GAD-positive (Buckmaster and Jongen-Relo, 1999) and somatostatin- and parvalbumin-immunoreactive interneurons are most reduced (Buckmaster and Dudek, 1997). Therefore, reduced granule cell inhibition and interneuron loss in the temporal dentate gyrus may be missed in studies of the septal dentate gyrus. Several workers have reported changes in the expression and function of $GABA_A$ receptors in epilepsy. There is an increase in membrane $GABA_A$ receptors in the dentate gyrus which is reflected in an increase in the efficacy of GABA in activating whole cell currents and increase in mIPSC amplitude (Otis et al, 1994; Gibbs et al, 1997; Nusser et al, 1998). As well as an increase in receptor density, $GABA_A$ receptor function is also altered in TLE. $GABA_A$ receptors on dentate granule cells become sensitive to blockade by zinc in contrast to $GABA_A$ receptors in controls which are zinc insensitive (Buhl et al, 1996; Gibbs et al, 1997). This is due to altered subunit

composition from receptors containing predominantly alpha1 and alpha2 subunits to receptors containing much higher levels of the alpha4 subunit (Brooks-Kayal et al, 1998). This potentially leads to a zinc-induced collapse of inhibition in the dentate gyrus and a breakdown of the barrier function of the dentate gyrus (Heinemann et al, 1992; Lothman et al, 1992; Buhl, 1996; Gibbs et al, 1997).

1.14 $GABA_B$ receptors

In 1980, a GABA receptor was identified that was markedly different from the classical GABA receptor (Bowery et al, 1980). It was activated by GABA but was not sensitive to the classical GABA receptor antagonists such as bicuculline and its action was independent of chloride. Its most striking characteristic was that the spasmolytic drug baclofen (β parachlorophenyl GABA), mimicked the effects of GABA at this receptor. Ligand-binding studies identified distinct attachment sites for baclofen on central neuronal membranes (Hill and Bowery, 1981). This previously unknown receptor was termed $GABA_B$ to differentiate it from the classical bicuculline-sensitive GABA receptor, which was in turn termed $GABA_A$.

$GABA_A$ receptors are ligand-gated ion channels and form part of the ionotropic receptor superfamily. $GABA_B$ receptors on the other hand, are coupled to G proteins (Wojcik and Neff, 1984; Karbon and Enna, 1985) and are thus classed as metabotropic receptors. They occur both pre- and post- synaptically. They inhibit adenylate cyclase activity, cause post-synaptic hyperpolarization and inhibit transmitter release.

1.14.1 Structure of the $GABA_B$ receptor

The $GABA_B$ receptor belongs to the G protein coupled receptor superfamily, which also includes metabotropic glutamate receptors and calcium sensing receptors. It is most similar to class C receptors which contain a large extracellular amino terminal domain. It contains a classic seven membrane spanning domain structure and an intracellular carboxy-terminal tail (Couve et al, 2000). The $GABA_{B1}$ subunit was cloned by Kaupmann at al (1997). However, the recombinant $GABA_{B1}$ subunit exhibited extremely low binding affinities for agonists when compared to wild-type $GABA_B$ receptors and the coupling to $GABA_B$ effector systems in heterologous cells was inefficient (Kaupmann et al, 1998). It was subsequently realised by Couve et al (1998) that the $GABA_{B(1)}$ protein was not trafficked to the cell membrane but remained associated with the ER. It was thus suspected that a trafficking protein might be required for functional expression (McLatchie et al, 1998). This hypothesis was proven when the second $GABA_B$ receptor subunit was discovered by Kaupmann et al (1998). Heterodimerisation between $GABA_{B(1)}$ and $GABA_{B(2)}$ is necessary to form a functional receptor because $GABA_{B(2)}$ trafficks $GABA_{B(1)}$ to the surface and $GABA_{B(2)}$ links to the G protein (Robbins et al, 2001). The $GABA_{B(1)}$ subunit is necessary for agonist activation (Margeta-Mitrovic et al, 2000; Calver et al, 2001; Galvez et al, 2000; Pagano et al, 2001). Thus, the agonist binds to a site on the $GABA_{B(1)}$ subunit which produces a conformational change in the protein complex that allows $GABA_{B(2)}$ to activate the G protein coupled signalling system.

1.14.2 $GABA_B$ receptor effector systems

$GABA_B$ receptors mediate their effects via the adenylate cyclase system and calcium and potassium ion channels (Hill, 1985; Gage, 1992). $GABA_B$ receptor activation is mediated by G proteins in the pertussis toxin-sensitive family $G_{i\alpha}/G_{o\alpha}$, especially $G_{i2\alpha}$ (Odagaki et al, 2000; Odagaki et al, 2001). In brain slices it has been shown that activation of $GABA_B$ receptors inhibits adenylate cyclase via G proteins and leads to a reduced level of intracellular cyclic AMP. Activation of presynaptic $GABA_B$ receptors suppresses calcium influx into presynaptic terminals (Isaacson and Hille, 1997), primarily via inhibition of P/Q-(Barral et al, 2000; Mintz and Bean, 1993) and N-type (Easter and Spruce, 2002) calcium channels. Postsynaptic $GABA_B$ receptors increase potassium conductance in neuronal membranes and may be associated with multiple types of potassium channels (Luscher et al, 1997).

1.14.3 Modulation of epileptiform activity by $GABA_B$ receptor agonists and antagonists

Drugs that act on $GABA_B$ receptors modulate seizure activity. However, they can be pro- or anti-epileptic depending on the model and seizure type. Baclofen suppresses epileptiform activity in hippocampal slices induced by high $[K^+]$ (Ault et al, 1986; Swartzwelder et al, 1986a), bicuculline (Ault et al, 1986), kainic acid (Ault et al, 1986), or stimulus trains (Swartzwelder et al, 1986a; Swartzwelder, 1986b). In vivo, baclofen prevents rapid amygdala kindling (Wurpel, 1994) and suppresses flurothyl- (Garant et al, 1993), pentylenetetrazol (De Sarro et al, 2000) and stimulus train- (Stringer and Lothman, 1990) induced seizures. Consistent with the notion that

$GABA_B$ activation is antiepileptic, the $GABA_B$ antagonist SCH 50911 is proconvulsive in rats undergoing ethanol withdrawal syndrome (Carai et al, 2002). In contrast, baclofen also has pro-epileptic properties under certain conditions. It induces spontaneous rhythmic sharp waves in hippocampal slices (Lewis et al, 1989). Baclofen can also induce ictal-like activity in hippocampal slices perfused with low [Mg^{2+}] or 4-aminopyridine (Dreier and Heinemann, 1991; Motalli et al, 1999; Swartzwelder et al, 1987; Watts and Jefferys, 1993; Avoli et al, 2004). $GABA_B$ receptor activation is also strongly implicated in the genesis of absence seizures (Hosford et al, 1992; Snead, 1992; Vergnes et al, 1997).

1.14.3 Altered $GABA_B$ receptors in epilepsy

Altered $GABA_B$ receptor function is implicated in epilepsy, although most work has been directed at the role of $GABA_B$ receptors in the pathogenesis of absence seizures. However, $GABA_B$ receptors undergo changes in TLE, in both human patients and experimental models. Haas et al (1996) measured paired pulse suppression of recurrent IPSPs (disinhibition) in rats two weeks after kainic acid induced SE and reported a downregulation of $GABA_B$ receptors in the polysynaptic recurrent inhibitory circuit in the dentate gyrus. They proposed that this is one mechanism that could induce an enhancement of dentate inhibition after seizures. Wasterlain et al (1996) showed loss of $GABA_B$ mediated slow inhibitory postsynaptic potentials (IPSPs) recorded from dentate granule cells following unilateral stimulation of the perforant path. Wu and Leung (1997) reported reduced paired pulse depression of IPSCs recorded in CA1 after kindling that persisted for at

least 21 days due to downregulation of presynaptic $GABA_B$ receptors. A decrease in efficacy of presynaptic $GABA_B$ receptors in glutamatergic terminals was shown in the basolateral amygdala after amygdala kindling (Asprodini et al, 1992). Kokaia and Kokaia (2001) reported changes in $GABA_B$ immunoreactivity in the hippocampus after kindling.

In addition to altered $GABA_B$ receptors in experimental epilepsy models, changes have also been identified in human patients with TLE. Munoz et al (2002) showed loss of $GABA_B$ staining on dentate granule cells in tissue from TLE patients. Billinton et al (2001) and Princivalle et al (2002) demonstrated altered binding to $GABA_B$ receptors in tissue from TLE patients using autoradiography. They demonstrated reduced $GABA_B$ density in CA1, CA2, CA3, hilus and dentate gyrus. However when corrected for cell loss, CA1 $GABA_B$ receptor expression appeared significantly upregulated. In addition they found increased affinity at CA3 and hilar $GABA_B$ receptors.

1.14.4 The role of $GABA_B$ receptors in absence epilepsy

Absence epilepsy is a condition characterised by generalised seizures that occur most commonly in childhood. During absence seizures, patients stare and cease normal activity for a few seconds, then return immediately to normal and have no memory of the event (Chang and Lowenstein, 2003). These seizures have a typical EEG pattern which consists of 3 Hz generalised spike-wave discharges. The mechanism that generates these seizures originates in the thalamocortical circuitry. It

was shown in the feline generalised penicillin model of epilepsy that both the cortex and the thalamus are needed to generate 3 Hz spike and wave seizures (Avoli and Gloor, 1982a,b). The same circuitry is also involved in the production of sleep spindles; these phenomena are related to absence seizures and occur in the same stages of sleep (Kellaway, 1985). The cellular elements involved in the evolution of these seizures and sleep spindles have been studied in detail (Steriade et al, 1993; McCormick et Bal, 1997). Thalamocortical relay cells receive input from various brain regions. They make excitatory projections to the cortex. Cortical neurons, in turn, have excitatory connections with the thalamus. The thalamic reticular nucleus (nRT) consists of a layer of almost pure GABAergic neurons and is located in the lateral part of the thalamus. It receives collaterals from the thalamocortical and corticothalamic axons. These collaterals excite the cells of the nRT which then project back to the thalamus and connect with other nRT cells via inhibitory synapses.

McCormick, Bal and collaborators developed a slice preparation from the lateral geniculate nucleus of the ferret (von Krosigk et al, 1993). This preserves the circuitry needed to generate spindle wave oscillations and absence-like 3 Hz rhythm. Release of GABA from nRT cells (known as perigeniculate cells, PGN, in this region of the thalamus) induces IPSPs in thalamocortical cells. Upon recovery from one of these IPSPs, the thalamocortical cells are capable of generating large calcium spikes on which are often superimposed a burst of action potentials. These action potentials in turn generate EPSPs onto GABAergic PGN cells. This triggers calcium

spikes in these cells with superimposed action potentials and thus the network oscillation continues. This is the type of oscillation that occurs in spindle wave activity; a normal part of sleep characterised by discharges occurring at a frequency of about 10 Hz. These studies in ferrets demonstrate that different oscillatory patterns can be produced in the thalamus depending on the manner in which GABAergic cells fire. Brief firing in PGN cells produces fast $GABA_A$ receptor mediated IPSPs in thalamocortical cells which sets the spindle wave frequency to 10 Hz. However, strong firing in PGN cells produces slow $GABA_B$ receptor mediated IPSPs in thalamocortical cells which changes the oscillation frequency to 3-4 Hz (Kim et al, 1997). Hence, $GABA_B$ receptors seem to play a key role in setting the oscillatory rhythm of the thalamocortical circuitry in absence seizures, and it is enhanced firing that causes the transition from sleep spindles to spike-and-wave discharges. How does this occur? It has been hypothesized that increased firing in the cortex causes increased firing in thalamic GABAergic cells, leading to increased GABA release and activation of $GABA_B$ receptors.

1.15 Are other receptors altered in TLE?

Several other receptor types are altered in the epileptic brain.

1.15.1 Metabotropic glutamate receptors (mGluRs)

It is likely that altered mGluR function plays a role in epileptogenesis (Doherty and Dingledine, 2001). Alterations in mGluR expression are observed both in

experimental epilepsy models and tissue from TLE patients. MGluR mRNA levels are altered in the hippocampus after kainic acid induced SE and results vary depending on the subunit studied and the cell type (Aronica et al, 1997). There was a downregulation of mGluR2 mRNA in dentate granule cells. Klapstein et al (1999) observed reduced perforant path sensitivity to mGluR group III agonists in kindled rats although Friedl et al (1999) found no significant change in the same model. Neugebauer et al (1997) reported an enhancement in group II sensitivity in the epileptic amygdala which was consistent with the findings of Doherty and Dingledine (2001) who demonstrated an increase in sensitivity to mGluR agonists in the SE-experienced dentate gyrus. Similarly, in epileptic human tissue mGluR1a (Blumcke et al, 2000) and mGluR4 (Lie et al, 2000) immunoreactivity is increased in the dentate gyrus.

1.15.2 Other receptor types

Several other receptor and ion channel abnormalities are associated with epilepsies. These include observations of altered expression or function in models and humans, and mutations in genes encoding for certain channels or receptors. There is some evidence that NMDA receptor expression and function is altered in epilepsy. Mathern et al (1998) found that NMDAR2b mRNA was upregulated in the dentate gyrus of rats after self-sustaining limbic SE and human TLE patients. Behr et al (2001) showed a transient NMDA receptor mediated facilitation of high frequency input in the rat dentate gyrus after kindling. Altered expression of NMDA subunits was also recently reported in pentylenetetrazol-kindled animals (Zhu et al, 2004).

Skyers et al (2003) observed an increase in opioid receptor labelling in the dentate gyrus following seizures. Also, changes in editing of mRNA encoding for AMPA receptor subunit GluR2 has been observed in hippocampi from epileptic humans (Vollmar et al, 2004). Enhanced expression of GluR1 flip AMPA receptor subunits has been observed in hippocampal astrocytes from epilepsy patients (Seifert et al, 2004). Recently, mutations in genes encoding for receptors and ion channels are being associated with naturally occurring human epilepsies. These include subunits of the $GABA_A$ receptor (Baulac et al, 2001), sodium channel (Wallace et al, 2001b), chloride channel (Haug et al, 2003), potassium channels (Eunson et al, 2000), calcium channels (Jouvenceau et al, 2001) and nicotinic acetylcholine receptors (De Fusco, 2000).

1.16 Are $GABA_B$ receptors a useful target for antiepileptic drugs?

Enhancing GABAergic inhibition is an effective way of treating epilepsy. This has been evident with treatments that act at $GABA_A$ receptors such as barbiturates and benzodiazepines. An alternative way of enhancing GABAergic inhibition and stopping seizures could be via $GABA_B$ receptors.

There has been suggestion that some antiepileptic drugs may exert their effect via $GABA_B$ receptors. Ng et al (2001) proposed that the antiepileptic drug gabapentin was a $GABA_B$ receptor agonist but this was subsequently disputed by Lanneau et al (2001) who were unable to replicate their results. Karlsson (1992) showed that $GABA_B$ agonists retarded amygdala kindling but antagonists accelerated it. It has also been suggested that CGP 36742, a $GABA_B$ receptor antagonist, may prove to be useful in the management of primary generalised absence seizures (Czuczwar and Patsalos, 2001). I asked if activation of $GABA_B$ receptors by the $GABA_B$ agonist baclofen could inhibit excitatory transmission and stop seizures. NB drugs that act at the $GABA_B$ receptor may have complex network effects because they are present both pre- and post-synaptically at the mossy fibre synapse and inhibit transmitter release at both inhibitory and excitatory synapses.

Another way of increasing $GABA_B$ receptor activation is by blocking GABA uptake and increasing ambient GABA concentration. I asked if the antiepileptic activity of the GABA uptake blocker tiagabine was due to increased GABA acting on $GABA_B$ receptors.

1.17 Tiagabine

This new antiepileptic drug was developed from the GABA uptake inhibitor nipecotic acid. Unlike nipecotic acid it can pass through the blood brain barrier because it is more lipid soluble than its parent compound. It is a potent selective inhibitor of the GABA transporter GAT-1. Its mode of action as an antiepileptic drug is not fully understood, but it is suspected that it may enhance inhibition via an increase in $GABA_A$ receptor mediated tonic inhibition and by increasing $GABA_A$ receptor mediated currents (Suzdak and Jansen, 1995). In addition, increased ambient GABA could activate $GABA_B$ receptors. Post-synaptic $GABA_B$ receptors are often positioned extrasynaptically and are only activated when GABA is simultaneously released from multiple synapses which leads to GABA spillover (Scanziani, 2000). Four GABA transporter (GAT) proteins have been cloned in both rats and humans: GAT-1, GAT-2, GAT-3 and BGT-1 (Borden, 1996). GAT-1 is the most prevalent GABA transporter in the rat forebrain and is predominantly found on presynaptic GABAergic terminals and glia. A decrease in clearance of synaptically released extracellular GABA has been observed in epilepsy, possibly due to reduced expression or function of GABA transporters (During et al, 1995; Patrylo et al, 2001). Tiagabine's anti-epileptic action is usually suggested to be due to an increase in *extracellular* GABA concentration. However, the time-course of the GABA transient in the synaptic cleft will also be affected, and by decreasing GABA uptake there is greater spillover of GABA on to extra-synaptic receptors. Increasing extracellular GABA does not necessarily increase GABAergic inhibition. Increasing

extracellular GABA can desensitize synaptic $GABA_A$ receptors which will result in reduced amplitude of $GABA_A$ receptor mediated currents (Overstreet et al, 2000). However, an increase in extracellular GABA can lead to an increase in tonic inhibition which is mediated by high affinity extrasynaptic $GABA_A$ receptors. Spillover of GABA may also enhance $GABA_B$ receptor mediated inhibition. Scanziani (2000) reported that blocking GABA uptake can result in activation of $GABA_B$ receptors by GABA released from a single interneuron which does not occur under baseline conditions. Activation of post-synaptic $GABA_B$ receptors may decrease excitability by hyperpolarising the post-synaptic neuron but there is also evidence that $GABA_B$ receptor activation can modulate oscillation frequency (Scanziani, 2000). Also, activation of post-synaptic $GABA_B$ receptors in the thalamus has been implicated in the generation of absence seizures (von Krosigk et al, 1993). A defect in GABA uptake was implicated in the pathogenesis of genetic absence epilepsy in a rat model (Richards et al, 1995; Sutch et al, 1999). Tiagabine can actually exacerbate absence seizures and induce absence status epilepticus (Coenen et al, 1995; Hosford and Wang, 1997). Tiagabine is an effective antiepileptic drug in several animal models. It stops status epilepticus in our self-sustained SE model. I asked if its antiepileptic action was partly due to $GABA_B$ receptor activation.

1.18 Summary of introduction

I investigated the role of $GABA_B$ receptors in temporal lobe epilepsy in two rodent models.

I asked the following questions:

1. Is $GABA_B$ receptor mediated heterosynaptic depression present at the adult rat mossy fibre –CA3 synapse?
2. Is it altered after status epilepticus?
3. Via what mechanisms is heterosynaptic depression altered after status epilepticus?
4. What is the source of GABA that mediates heterosynaptic depression?
5. Is baclofen antiepileptic in our models of status epilepticus?
6. Is the action of the antiepileptic drug tiagabine mainly mediated by $GABA_B$ receptors?

Chapter 2: General Methods

2.1 Epilepsy Models

I used adult male Sprague Dawley rats in all experiments. They were kept under a 12 hour light-dark cycle with free access to water and a normal laboratory diet (SDS R and M number 1 expanded, Scientific Dietary Services, Witham, Essex, UK). All animal procedures followed the Animal (Scientific Procedures) Act, 1986.

2.1.1 Perforant path stimulation

I selected this model for several reasons. First, the induction of self-sustaining status epilepticus is highly reproducible (Holtkamp et al, 2001). Second, in our laboratory, the seizure severity is similar in each animal and there is a minimal mortality rate. Third, the EEG can be recorded during and after stimulation via the implanted electrodes. Thus status epilepticus can be confirmed electrographically and monitored during administration of antiepileptic drugs.

2.1.1.1 Electrode preparation

Bipolar stimulating electrodes were made by twisting two 125 μm Teflon coated stainless steel wires together. The tips of the electrodes were separated by approximately 0.5mm. A single 250μm stainless steel wire was used as the recording electrode and a silver wire was used for the earth electrode.

2.1.1.2 Anaesthesia

Male Sprague-Dawley rats (270-330 g) were placed in an induction chamber and administered 4% halothane in O_2. When they became laterally recumbent and unresponsive to a foot pinch they were removed from the chamber, and placed in a stereotaxic frame where anaesthesia was maintained with 1-2% halothane in O_2 at a flow rate of 2 litres / minute.

2.1.1.3 Electrode placement

Two full-thickness holes were drilled into the skull using a drill mounted on a stereotaxic frame for placement of the recording (coordinates 2.5 mm lateral, 4 mm caudal from bregma) and stimulating (coordinates 4.4 mm lateral, 8.1 mm caudal from bregma) electrodes. Three further holes were then drilled for placement of skull screws that would be used for anchoring of the headstage later. A bent hypodermic needle was used to tear the dura if it had not been penetrated by the burr. Three screws were placed in the anchoring holes. The silver wire earth electrode was wound around one of the screws to anchor it, and then positioned subcutaneously. The bipolar stimulating electrode was implanted in the right hemisphere, and advanced to a depth of 4 mm into the angular bundle to stimulate the perforant path. The monopolar recording electrode was implanted stereotaxically into the right hippocampus and lowered through the skull hole until it touched the brain parenchyma. It was then slowly lowered into the parenchyma while test shocks were applied to the stimulating electrode and any response observed on the oscilloscope. It was lowered until the slope of the population excitatory post

synaptic potential (EPSP) was maximal as described by Errington et al (1987). Once all the electrodes were in place and a fEPSP could be recorded, dental cement was applied around the electrodes and screws to anchor them in place. The gold connectors of the three electrodes were then placed into a plastic headstage. The headstage itself was also held in place by more dental cement. A piece of tape was placed over the holes of the headstage as a protective barrier. The animals were allowed to recover from anaesthesia. Seven days later the perforant path was electrically stimulated with 4-5 mA, 50 μs monopolar pulses at 20 Hz for two hours; this induced self-sustaining SE that was terminated after 2 hours with propofol (50 mg/kg i/p) or diazepam (10 mg/kg i/p). In some experiments baclofen (5-10 mg/kg) (Tocris Cookson), tiagabine (80 mg/kg) (gift from Novo Nordisk) or SCH50911 (40 mg/kg) (Tocris Cookson) were given.

Animals were killed 24 hours after SE with an overdose of pentobarbitone (500 mg/kg i/p).

2.1.2 The pilocarpine model

The perforant path model of status epilepticus was already in use in the laboratory. Stimulation of the perforant path evokes population spikes in granule cells and epileptiform discharges and is likely to cause excessive glutamate release. This leads to hippocampal damage which is similar to that seen in human patients with epilepsy. There are many different models of epilepsy available and changes in these models are sometimes model specific. It can therefore be difficult to know the relevance of some of these changes to human epilepsies. I therefore decided to compare two different models of status epilepticus to ensure that any changes

observed were not model specific. The pilocarpine model of seizures and epilepsy is one of the most commonly used chemical models. Pilocarpine is a muscarinic agonist and causes seizures via activation of the M1 muscarinic acetylcholine receptor (Hamilton et al, 1997; Berkeley et al, 2002). The pilocarpine model has both clinical and neuropathological features similar to human temporal lobe epilepsy (Turski et al, 1987; Cavalheiro, 1995) and is widely used by several different groups. This means that my data can be put into the context of the work of others. Muscarinic agonists suppress a voltage dependent tonically active potassium current known as the M current (Brown and Adams, 1980). Blocking the M current causes membane depolarisation and increases the probability of firing (Hamilton et al, 1997). During pilocarpine-induced seizures excessive glutamate is released which stimulates continuous release of acetylcholine and neuronal damage induced by NMDA receptor activation (Smolders et al, 1997). Since this method of seizure induction is different from that of perforant path stimulation but still results in histopathological changes similar to humans with temporal lobe epilepsy, I set out to develop this method in our laboratory.

2.1.2.1 Development of the pilocarpine model in our laboratory

Slight variations in methodologies have been used. The dose of pilocarpine used is variable but is usually in the range of 310-380 mg/kg (Churn et al, 2000; Brooks-Kayal et al, 1998; Mello et al, 1993; Covolan and Mello, 2000a,b; Kobayashi and Buckmaster, 2003). In most studies a peripheral muscarinic antagonist such as scopolamine methyl nitrate (or bromide) or atropine is given 30 minutes prior to

pilocarpine injection. This is claimed to reduce mortality because it reduces peripheral cholinergic side effects such as hypersalivation, increased secretions in the respiratory tract and hypermotility of the gastro-intestinal tract. A protocol of repeated low-dose pilocarpine injections has also been tried (Glien et al, 2001). Also, lithium can be given prior to pilocarpine adminstration which markedly reduces the dose of pilocarpine necessary to induce SE (Clifford et al, 1987; Honchar et al, 1983). Lithium may alter the response to pilocarpine by several mechanisms: decreasing noradrenaline or dopamine release, increasing acetylcholine release, altering muscarinic binding, or altering inositol phospholipid second messenger systems. I decided to use pilocarpine without the addition of lithium to reduce the complexity of any pathological effects of the drugs themselves. I selected my initial protocol by reviewing the literature and selecting the methods used most commonly (Churn et al, 2000; Covolan et al, 2000a,b; Brooks-Kayal et al, 1998; Arida et al, 1999; Kobayashi and Buckmaster, 2003). I defined the onset of status epilepticus as the appearance of Stage 3 (Racine, 1972) seizures (see General Introduction) followed by continuous clinically detectable seizure activity. Drugs were dissolved in 0.9 % sterile saline and were administered via the intraperitoneal route unless stated otherwise. I modified the initial protocol to reduce the mortality rate while maintaining a high percentage of status epilepticus induction.

My initial protocol consisted of the following:

Scopolamine methyl nitrate (Sigma) was given intraperitoneally at 1mg/kg. Thirty minutes later, I administered pilocarpine at 350mg/kg intraperitoneally. I monitored

the evolution and severity of the seizures. Pilocarpine at 350mg/kg resulted in prolonged generalised seizures in the first animal tested. I therefore reduced the pilocarpine dose to 325mg/kg which was still sufficient to induce seizures in all animals.

The following modifications were used to reduce the mortality rate:
Each rat was given two injections of scopolamine : one half an hour before the pilocarpine injection and one half an hour afterwards. This reduced overt signs of peripheral muscarinic effects such as piloerection, excessive salivation and increased intestinal activity (frequent defaecation or diarrhoea). The animals were given 5 ml of saline subcutaneously immediately after SE and offered fluid gel when they were able to swallow and sufficiently recovered to take fluids *per os*. Later they were offered soaked food pellets. I used minimal restraint when handling the rats and used insulin syringes to administer drugs which reduced the trauma of injection. I allowed them to adjust to their new environment for a minimum of twenty minutes before starting each experiment. Paper linings were placed in the cages to prevent inhalation or choking on sawdust which sometimes appeared to cause respiratory distress.

After these modifications had been made, the mortality rate was approximately 10%. I therefore used the final protocol below:

Table 2.1 Final protocol for pilocarpine model

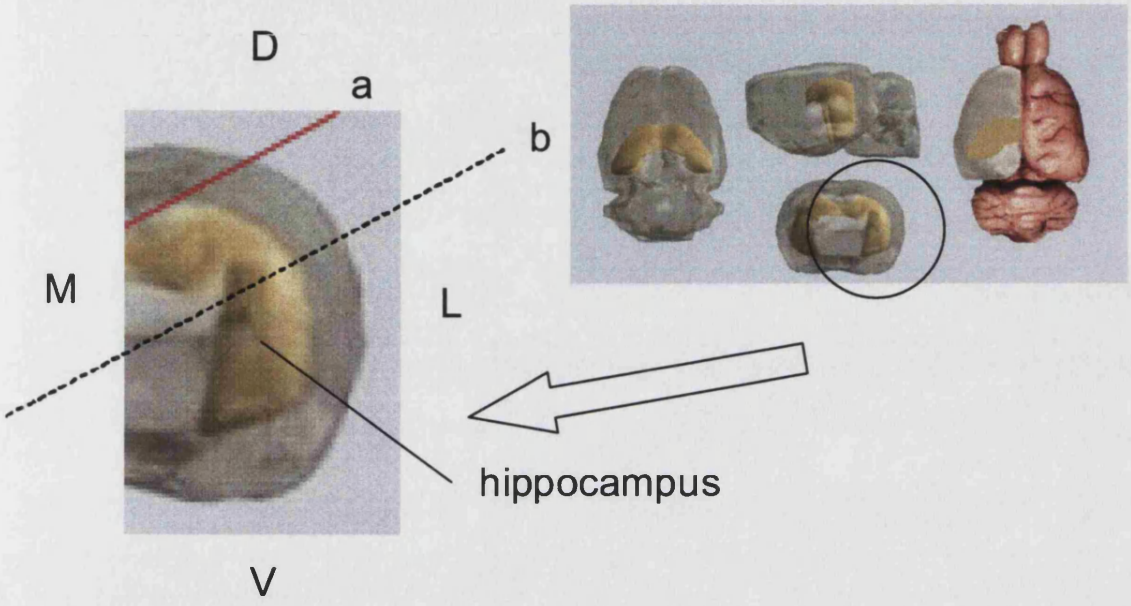
Time (mins)	Drug	Dose	Concentration (mg/ml)
0	Scopolamine methyl nitrate (Sigma)	1 mg/kg	1
30	Pilocarpine hydrochloride (Sigma)	320 mg/kg	320
60	Scopolamine methyl nitrate (Sigma)	1 mg/kg	1
90 mins after start of SE	Diazepam (Phoenix Pharma)	10 mg/kg	5
90 mins after start of SE	0.9 % sterile saline (s/c)	5 ml	

2.2 Slice preparation

In all experiments, rats were killed with an overdose of pentobarbitone (500 mg/kg) i/p. Epileptic animals were killed either 24 hours or three weeks after status epilepticus. The brain was removed in < 1 minute and immersed in oxygenated ice-cold sucrose solution which contained (in mM), NaCl 87, KCl 2.5, $MgCl_2$ 7, sucrose 75, $CaCl_2$ 0.5, NaH_2PO_4 1.25 and glucose 25. This was used because the tissue preservation appeared to improve when compared to other slicing solutions such as Ringer or choline based solution (personal communication from other laboratory members). In order to perform many of the *in vitro* experiments, two separate groups of mossy fibre axons needed to be stimulated and recorded from simultaneously in each slice. Mossy fibres run transversely through the hippocampus. The angle of slice preparation has to be precise; otherwise mossy fibre axons will be transected, making identification of two pathways from granule cells to CA3 unfeasible. Therefore, slice preparation at the correct angle is imperative for these experiments.

Two methods of hippocampal slice preparation were tried. The aim was to prepare transverse hippocampal slices with as many intact mossy fibres as possible.

Figure 2.1: Whole brain slicing method (D = dorsal, L = lateral, M=medial, V=ventral), adapted from web resource: http://synapses.mcg.edu/index.asp



In the first method (Fig. 2.1), each hemisphere was removed from the sucrose solution and a cut (a, red line) was made as illustrated with a scalpel blade. The cut surface of the hemisphere was glued onto the cutting stage. Slices of 400 µm were prepared using a Leica vibratome. Slices were cut along the plane of the line, b. The slices collected in the middle of the hippocampus are transverse. As each slice was prepared, the hippocampus was dissected using two hypodermic needles. With this slicing method, I frequently obtained slices in which two mossy fibre pathways could not be identified. In this situation fEPSPs could be recorded if the axons were stimulated in *s. lucidum*, but not in the dentate granule cell layer. This suggested that

the axons had been severed during the slicing procedure. I therefore changed to the following method.

After the whole brain had been removed and placed into ice-cold sucrose solution, each hippocampus was removed from its hemisphere using spatulas to peel it away from the rest of the temporal cortex. It was gently straightened and placed in a previously prepared agar block that had had two grooves which were cut slightly wider than the width of the hippocampi. Transverse slices at 400 μm were then obtained using the Leica vibratome. This technique yielded slices with two mossy fibre pathways more frequently.

2.3 Electrophysiology

Transverse hippocampal slices (400 μm thick) were obtained from control rats and rats following SE as described above. They were stored in an interface chamber containing Ringer solution for at least 1 hour prior to transfer to a submersion recording chamber. The storage and perfusion solution contained (in mM) NaCl (119), KCl (2.5), $MgSO_4$ (4), $CaCl_2$ (4), $NaHCO_3$ (26.2), NaH_2PO_4 (1), and glucose (11), and was gassed with 95% O_2/5% CO_2 (23-25 °C). The CA3 region, which is where I was recording, is prone to spontaneous epileptiform activity *in vitro*, and thus I used high concentrations of divalent cations to increase the "charge screening" of axons (Guth and Drescher, 1990). This high concentration of cations was used in both control and epileptic slices.

2.3.1 Field potentials

Extracellular changes in potential that arise due to synaptic activity (field potentials) can be recorded *in vivo* and also in slice preparations. I recorded field potentials in acute hippocampal slices in order to measure synaptic activity at the mossy fibre to CA3 pyramidal cell synapse. Field potentials consist of a presynaptic component (the fibre volley) and a post-synaptic component (the field excitatory post synaptic potential (fEPSP). If mossy fibres are stimulated in the dentate granule cell layer by applying a current across a bipolar electrode and extracellular potential is measured in *s.lucidum* of CA3 (i.e. the dendritic layer where mossy fibres terminate on the proximal dendrites of CA3 pyramidal neurons), then the fEPSP is recorded as a negative potential (Fig. 2.1). Mossy fibres often have a large complex fibre volley (reflecting depolarization of presynaptic axons) that contaminates the initial part of the fEPSP. For this reason, I measured amplitude of the field potential rather than the slope; measurement of the fEPSP slope is often used, as the initial slope is not contaminated by population spikes or IPSPs. I also, at the end of all experiments, applied the AMPA/kainate receptor antagonist NBQX to abolish the fEPSP, leaving the fibre volley. I was then able post-hoc to subtract the fibre volley from the recorded field potentials before analysis, giving me a more accurate measure of fEPSP amplitude.

Field potentials were recorded using glass microelectrodes (resistance ~1 MΩ) filled with perfusing solution. A Digitimer DS3 isolated constant current stimulator

was used to apply electrical stimuli to the slice through a bipolar stimulating electrode. I constructed the electrodes by positioning two tungsten electrodes (8-10 MOhm resistance) (FHC) at approximately 30° to each other with the tips as close together as possible without touching, which was achieved under a dissecting microscope. They were then glued together using dental cement which was arranged in such a way so that the finished bipolar electrode could be inserted into the electrode holder.

The following drugs were used to block $GABA_B$, AMPA/kainate and group II metabotropic glutamate receptors (mGluRs); CGP52432 (5 μM) or SCH50911 (20 μM), NBQX (50 μM), and LY341495 (500 nM). NO711 (20 μM) was used to block GABA uptake. The $GABA_B$ receptor agonist baclofen (0.1 -10 μM) was used to determine $GABA_B$ receptor sensitivity. (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)-glycine (DCG-IV) was used to activate group II metabotropic glutamate receptors. All drugs were obtained from Tocris, except for DCG-IV and NO-711 (Sigma).

2.4 Immunogold labelling

Animals used for immunogold labelling were perfused over 20 minutes with 4% paraformaldehyde while under deep pentobarbitone anaesthesia. Immunogold labelling of GABA was examined at the ultrastructural level using electron microscopy to measure GABA within the mossy fibre terminals. This is much more specific than using other immunocytochemical methods at light microscopic level as

the mossy fibre terminals can be precisely identified. Light microscopy cannot easily distinguish between labelled GABA in mossy fibres from that in the extracellular space and/or in other terminals. A modified Timms stain was used to confirm the identity of mossy fibres. Samples were incubated with a primary rabbit antibody raised to GABA (Sigma). The sample was then incubated with a secondary antibody which was goat anti-rabbit IgG coupled to 10 nm gold particles (Sigma). Electron microscopy was used to reveal the localization of the gold particles. The electron microscopy and results described in this part of the thesis were obtained by Ruth Fabian-Fine. The methods that she used are described in more detail in Chapter 5.

2.5 Theory of autoradiography

How can we analyse receptors by radioligand binding?

Drug + Receptor $\rightleftharpoons$ drug-receptor complex $\rightleftharpoons$ active drug receptor complex

affinity *efficacy* ⇩

Response

As can be seen from the reaction scheme above, the binding of a drug to a receptor is the first step in the pathway that allows transduction of a signal from the outside to the inside of a cell. In autoradiography, a radioactive ligand can be used to measure binding to a receptor. How was a suitable ligand selected? Antagonists tend to be used more commonly than agonists because they provide the basis for a more robust assay. This is because agonist binding can be influenced profoundly by local concentrations of GTP and other nucleotides, and by mono- or di-valent cations and

pH (Wharton and Polak, 1993). Often, antagonists of higher affinity than their corresponding agonists have been synthesized by the pharmaceutical industry. Care should be taken to select a ligand with an appropriate affinity. The off-rate should not be so slow that problems are encountered with equilibrium binding, or so fast that the ligand-receptor complex dissociates before the complex can be measured. The equilibrium dissociation constant (K_D) should be in the range 50 pM to 25nM. The ligand selected was [^{3}H]-CGP 62349 , a $GABA_B$ receptor antagonist that had been used successfully in Professor Bowery's laboratory previously and its use is described in detail in Chapter 7. The choice of radioisotope depends on such factors as half life of decay, chemical stability, solubility and cost. ^{3}H and ^{125}I are the most commonly used isotopes.

The concept of specific and non-specific binding is important. We measured specific binding as follows: the preparation was incubated with radioligand alone. This yielded a measurement of total binding. Similar incubations were performed in parallel with the same concentration of radioligand but also in the presence of a saturating concentration of an unlabelled competing ligand. This yielded a measurement of non-specific binding. Total minus non-specific binding was then calculated which provided specific binding for $GABA_B$ receptors. For the displaceable binding to equate with binding to a specific receptor, the displacing agent needs to have binding specificity for the receptor in question. Once the radioligand had bound to the receptors, how was the bound ligand detected? A radiosensitive film was exposed to the preparation, and the energy of radioactive

decay converted silver halide crystals to free silver. Tritium is a very weak β emitter. The film that I used, Hyperfilm ^{3}H, consists of a clear plastic sheet, with emulsion on one side, with no protective antiscratch layer. This allowed the weak emission to be detected. The images obtained using radiosensitive films could then be used to obtain quantitative data regarding the receptor binding. The concept behind this is that the film optical density can be measured in terms of known concentrations of tissue radioactivity. Darker areas on the film have higher binding than lighter areas. The specific methods used will be described in Chapter 7.

Chapter 3: Is $GABA_B$ receptor mediated heterosynaptic depression present at the adult rat mossy fibre – CA3 synapse?

3.1 Introduction

Hippocampal mossy fibres are sensitive to several neurotransmitters that depress transmitter release via action on their presynaptic receptors, including glutamate acting on metabotropic glutamate receptors (Kamiya et al, 1996), GABA acting at $GABA_B$ receptors (Min et al, 1998; Vogt and Nicoll, 1999), and endogenous opioids acting at opioid receptors (Weisskopf et al, 1993). What is the adaptive significance of the profound modulation exhibited at the mossy fibre synapse? Hippocampal principal cells are highly vulnerable to excitotoxicity (Meldrum, 1993). Prolonged seizure activity such as that induced by pilocarpine or kainate injection, causes damage to pyramidal neurons in both CA1 and CA3 (Covolan and Mello, 2000). Humans with temporal lobe epilepsy lose hippocampal principal cells (Foldvary et al, 1999) as part of the pattern of cell loss known as hippocampal sclerosis. Presynaptic inhibitory phenomena acting at the mossy fibre synapse could potentially reduce excitatory input into CA3 and minimise damage to these cells. Presynaptic inhibition at this synapse provides dynamic control of this strong input which could be very important in gating seizure activity into the hippocampus proper.

Second, profound modulation at the mossy fibre synapse could result in a sparse dynamic input to CA3 pyramidal neurons. Why might CA3 require a sparse input? It has been proposed that CA3 acts as an auto-associative memory. This is a way of storing memories that are represented by a pattern of neural activity. Stored memories can then be retrieved when the network is presented with a fragment of one of the memories by a process known as completion (Treves and Rolls, 1992). What properties would CA3 need to provide this function? A sparse but strong input is required to force CA3 cells into a pattern of activity that is relatively independent from information from the many collateral inputs within CA3 (Treves and Rolls, 1992). Mossy fibres potentially have the properties necessary to provide this strong and sparse input. Characteristics suggesting that they provide a *strong* input are: they have large synaptic terminals, form synapses on CA3 cell proximal dendrites, express marked frequency facilitation and large mean EPSP size. These properties suggest that mossy fibre synapses have a higher release probability than most other cortical synapses (Henze et al, 2000) and hence provide the strong input that would be required. They have also been described as conditional detonators by Henze et al (2002) meaning that they are capable of causing CA3 cells to fire depending on their firing frequency. The profound presynaptic modulation of mossy fibres could provide the *sparseness* required for CA3 to exhibit this form of memory.

One form of presynaptic modulation that occurs at the mossy fibre synapse is heterosynaptic depression. This arises when transmitter is released from one pathway and depresses transmission in an adjacent pathway. Studies in juvenile guinea-pigs have shown that this phenomenon is mediated both by group II

metabotropic glutamate receptors and by $GABA_B$ receptors (Min et al, 1998; Vogt and Nicoll). It is potentially an extremely important phenomenon because it provides a gate to excitatory input into the hippocampus proper. It could thus potentially prevent damage to the vulnerable hippocampal principal cells by halting propagation of seizure activity through the hippocampus proper. I therefore asked: is heterosynaptic depression present at the adult rat mossy fibre synapse, and is it mediated by the same receptor types as in the guinea-pig?

3.2 Methods

Acute hippocampal slices were prepared from adult control Sprague-Dawley rats, as described in Chapter 2. A recording electrode, with resistance of approximately 1MΩ, filled with Ringer solution identical to the extracellular recording solution (see Chapter 2: General Methods), was placed in *stratum lucidum*, which could be visualised as a pale band between *stratum pyramidale* and *stratum radiatum* of CA3 (Fig. 3.1).

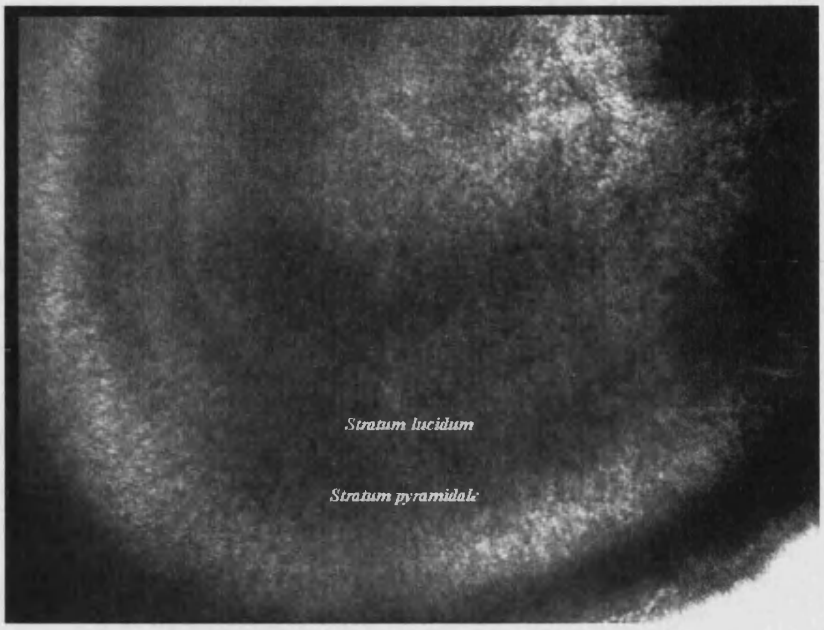


Figure 3.1: Low power photomicrograph (x 30 magnification) of hippocampal slice, demonstrating the different strata.

Two bipolar tungsten stimulating electrodes were positioned in the dentate granule cell layer and 0.5-1 mA pulses (80 μs duration) were applied with constant current stimulators. Stimuli were applied to one electrode at 0.2 Hz when searching for mossy fibre fEPSPs. If no response was observed, the depth and position of the recording electrode within *s.lucidum* was altered, and the stimulating electrode was systematically positioned along *s.granulosum* of the dentate gyrus until a response was found (Fig. 3.2).

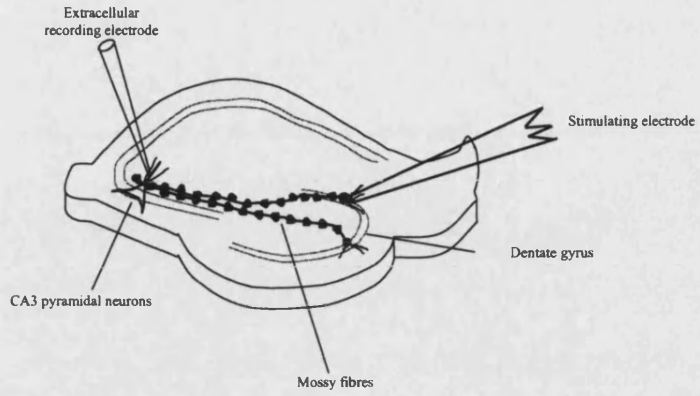


Figure 3.2: Mossy fibre field potentials were recorded in acute hippocampal slices in s.lucidum using this electrode arrangement. Stimulating electrodes were positioned systematically at different points in s.granulosum until fEPSPs could be elicited from each of them. The recording electrode was placed in stratum lucidum.

Once a putative mossy fibre fEPSP was found the stimulus intensity was increased (within a range of 0.5-1 mA) until the amplitude did not increase further. The stimulation was then reduced to the minimum necessary to achieve the maximal field potential amplitude. Typically, mossy fibre synapses exhibit marked frequency facilitation (Salin et al, 1996) and responses show a marked reduction in amplitude in the presence of (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)-glycine (DCG-IV) (Kamiya et al, 1996). I measured frequency facilitation and sensitivity to DCG-IV (1 μM), to test whether the response had characteristics of mossy fibre synapses. Once I had identified one putative mossy fibre pathway, I positioned a second stimulating electrode in *stratum granulosum* of the dentate gyrus, as far as way as possible from the first stimulating electrode. I then systematically moved the electrode through *s.granulosum* until another putative mossy fibre fEPSP emerged. Sensitivity to DCG-IV was measured in both pathways at the end of each experiment to ensure

they both had characteristics typical of mossy fibres. I then used a two pathway stimulation protocol to investigate whether heterosynaptic depression could be induced.

3.3 Results

In 8 slices from different control animals fEPSPs showed marked short-term frequency-dependent facilitation which was observed as an increase in amplitude of at least twice baseline when the stimulation frequency was increased from 0.05 Hz to 1 Hz (Fig. 3.3 A,B,C,D). In all experiments, I also confirmed that they showed a reduction to less than 30% of the baseline amplitude in the presence of DCG-IV (1 μM) (Fig. 3.3 A,B,C,D). Responses were rejected if they did not exhibit these two criteria, however it was extremely rare to record a fEPSP that did not show these two properties.

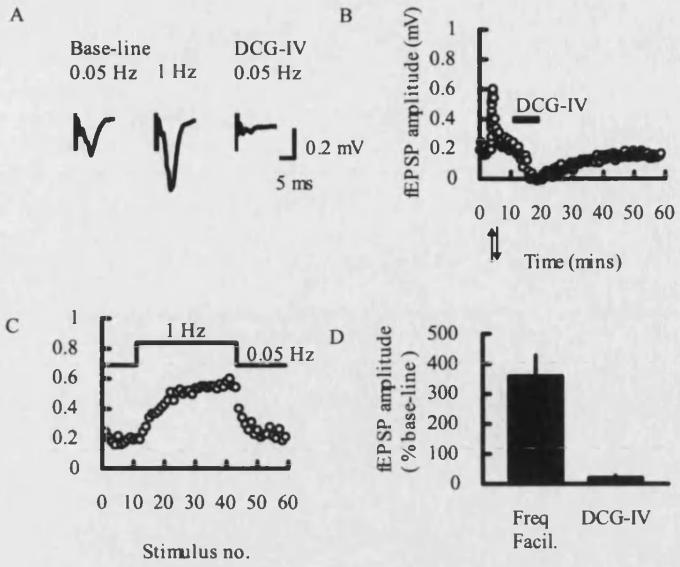


Figure 3.3: Identification of mossy fibre field potentials. I identified mossy fibre fEPSPs by demonstrating marked frequency facilitation and sensitivity to the Group II metabotropic glutamate receptor agonist DCG-IV. A, fEPSPs (traces = averages of 5 trials, taken from experiment shown in B) recorded in stratum lucidum whilst stimulating in stratum granulosum of the dentate gyrus at a baseline frequency of 0.05 Hz, 1 Hz and at 0.05 Hz in the presence of the group II metabotropic glutamate receptor DCG-IV (1μM). B, example of an experiment demonstrating the identification of mossy fibre fEPSPS by frequency facilitation (arrows show change in stimulation frequency from 0.05 Hz to 1 Hz) and DCG-IV sensitivity. C: same experiment as shown in B showing effect of increase in frequency plotted against stimulus number. D, Summary data (n = 8). The fEPSP amplitude increased to at least 200 % of baseline (20^{th} / 1^{st} response) when the stimulus frequency was increased from 0.05 Hz to 1 Hz, and the metabotropic glutamate receptor agonist DCG-IV (1 μM) decreased fEPSP amplitude to less than 30% of baseline.

Mossy fibre responses typically show paired pulse facilitation when two stimuli are applied to the same mossy fibre pathway (Salin et al, 1996). I used this property to confirm that the two pathways that I had identified were separate. Once I had found two mossy fibre fEPSPs I tested whether the fEPSP elicited in one pathway was facilitated by a preceding stimulus to the other pathway (Fig. 3.4). If I observed any facilitation I re-positioned the stimulating electrodes until no facilitation was

observed (mean cross-facilitation of 4 ± 3 % n = 6).

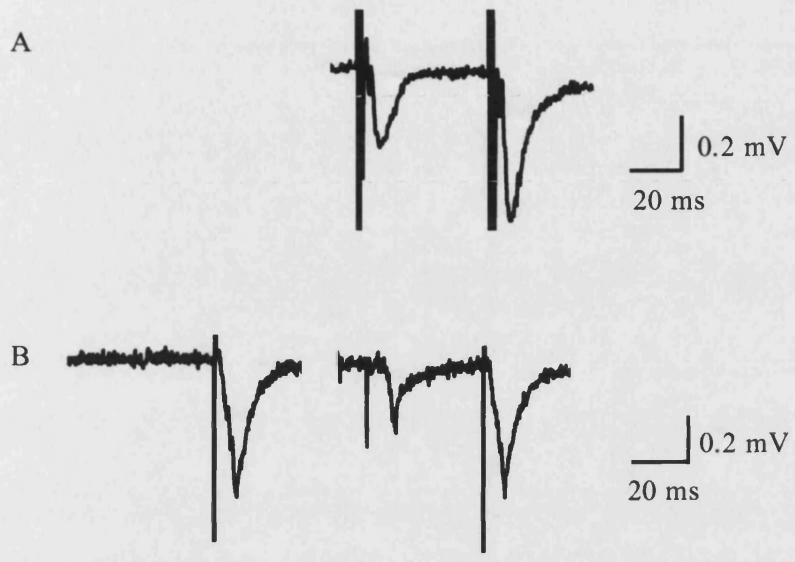


Figure 3.4: Mossy fibre fEPSPs exhibit paired pulse facilitation when two consecutive stimuli are applied to the same pathway but not when two different pathways are stimulated 40 ms apart . A, Two consecutive fEPSPs induced by stimulating the same mossy fibre pathway 50 ms apart, demonstrating marked facilitation of the fEPSP. B, The left trace is a mossy fibre fEPSP induced by a single stimulus applied in stratum granulosum of the dentate gyrus. The right trace shows the response from the same group of mossy fibres, preceded by a separate mossy fibre fEPSP induced by stimulating a different region of the dentate gyrus in the same slice 40 ms previously. The preceding fEPSP does not cause an increase in amplitude of the original response, demonstrating an absence of facilitation.

After I had identified two separate mossy fibre pathways, I used the following stimulation protocol to induce heterosynaptic depression. Single stimuli were applied to the "test" pathway at a frequency of 0.05Hz. Three hundred milliseconds before every 10^{th} stimulus a train of 5 stimuli at 50Hz was applied to the "conditioning" pathway (Fig. 3.5).

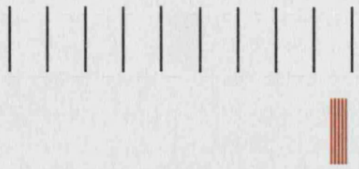


Figure 3.5: A schematic to show the stimulation protocol.
The black lines represent a cycle of stimulations at a frequency of 0.05 Hz applied to the test pathway. The red lines represent the conditioning train (5 pulses at 50 Hz) applied to the conditioning pathway which precedes every 10th low frequency stimulus.

I used a train of this frequency and duration because first, dentate granule cells fire in short bursts at high frequencies *in vivo* (Jung and McNaughton, 1993) and so this is a physiologically relevant stimulus. For example, Henze et al (2002) recorded trains of 26 Hz in dentate granule cells of awake mice. Second, Vogt and Nicoll (1999) could induce a heterosynaptic depression with a train of the same length and frequency in slices from guinea-pigs and Min et al (1998) observed a depression when using a train of only 20 Hz. When the train was applied, a marked facilitation of the induced response was seen (Fig. 3.6). This indicates that a large amount of transmitter is likely to be released during this stimulus.

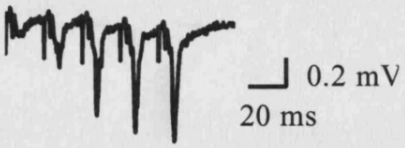


Figure 3.6: Mossy fibre fEPSPs recorded during application of a train of 5 stimuli at a frequency of 50 Hz. Marked facilitation was observed during the train.

I measured the amplitudes of the fEPSPs elicited by application of the test stimuli. The magnitude of heterosynaptic depression was calculated by comparing the amplitude of every 10th fEPSP recorded (conditioned fEPSPs or $fEPSP_C$) to the average amplitude of the nine preceding fEPSPs (unconditioned fEPSPs or $fEPSP_U$). The fEPSP ratio was calculated as $fEPSP_C/fEPSP_U$.

At the end of each experiment, I applied the AMPA/kainate receptor antagonist NBQX (50 μM) in order to record the remaining presynaptic fibre volley, which was then subtracted from the fEPSP prior to analysis of the results (Fig. 3.7). A high concentration of NBQX was used to ensure that all AMPA and kainate receptors were blocked (Bortolotto et al, 1999; Bureau et al, 2000).

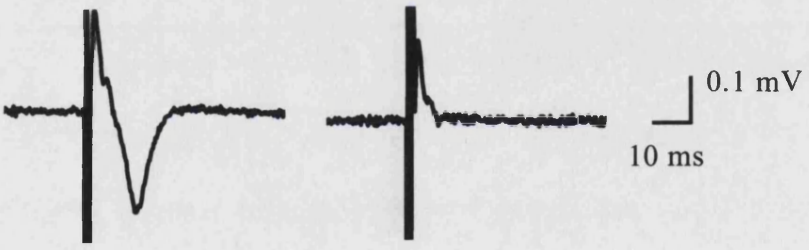


Figure 3.7: At the end of each experiment I applied the AMPA/kainate receptor antagonist NBQX (50 μM) in order to record the remaining presynaptic fibre volley. The trace on the left is a mossy fibre fEPSP recorded in naloxone only and the trace on the right shows the remaining stimulus artefact and presynaptic fibre volley in the presence of NBQX. (Each trace is an average of 5 trials stimulated at 0.05 Hz).

Once two separate pathways had been identified, I used the two pathway stimulation protocol (Fig. 3.5) to induce heterosynaptic depression as described in the methods. Heterosynaptic depression, expressed as (1-fEPSP ratio) x 100 %, was observed in

all slices from adult control rats (20 ± 7 % depression, n = 8 slices from different animals, Fig. 3.8).

What mediates this heterosynaptic depression ?

Weisskopf et al (1993) described a heterosynaptic depression mediated by endogenous opioids released from mossy fibres although the frequency of stimulation required to induce the release of opioids was much higher than that used in my protocol. Nevertheless, I applied the opioid antagonist naloxone (10 μM) during the same stimulus protocol and calculated the magnitude of depression, to determine if there was an opioid mediated component to the depression. Naloxone did not significantly change the magnitude of depression (depression without naloxone 20 ± 7 %, depression with naloxone 21 ± 7 %, n = 8, p = 0.9 paired t test Fig. 3.8A,B), suggesting that opioids are not involved in the depression that I observed. I subsequently did all two pathway experiments in the presence of naloxone to ensure that opioid receptors were blocked. I then applied the $GABA_B$ receptor antagonists, SCH50911 (Bolser et al, 1995) or CGP52432 (Lanza et al, 1993) while applying the same stimulation protocol. Bath perfusion of $GABA_B$ receptor antagonists (20 μM SCH50911, n = 6, or 5 μM CGP52432, n = 2) completely abolished heterosynaptic depression in all cases (0 ± 6 % depression, $p < 0.001$ for comparison with baseline, Fig. 3.8A,B).

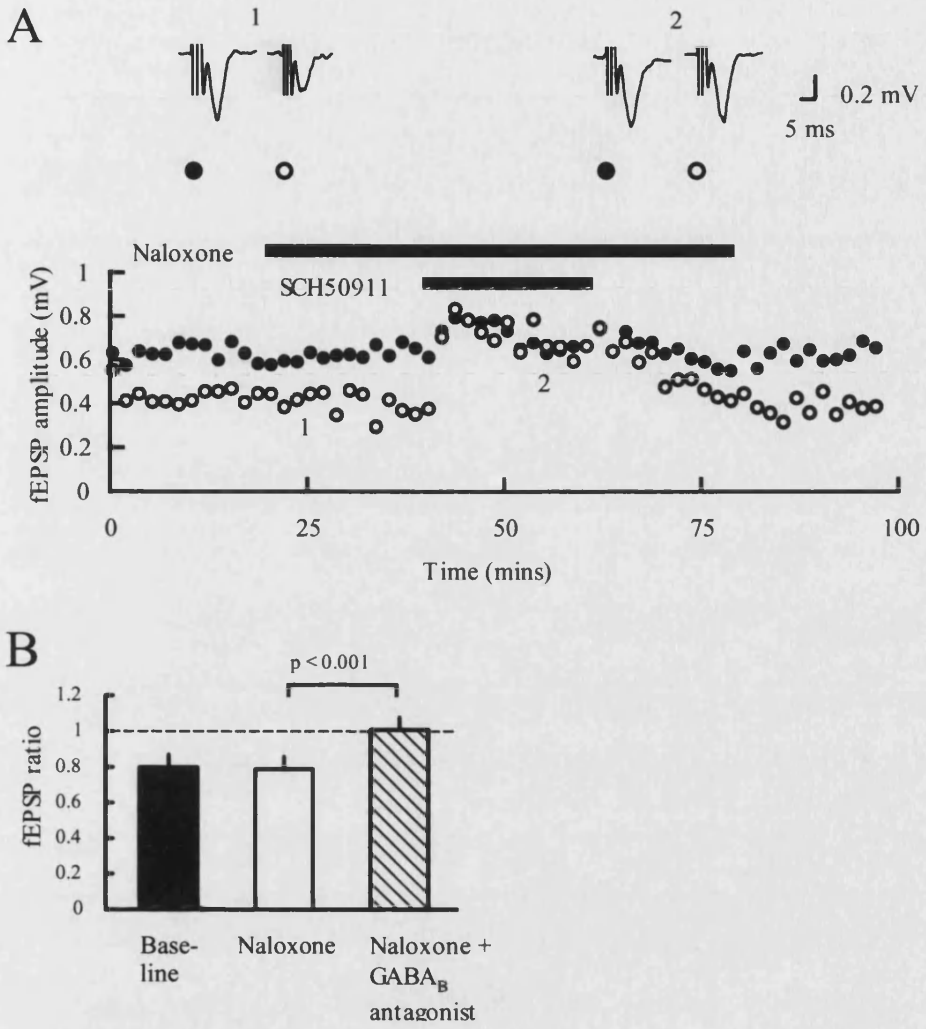


Figure 3.8: Heterosynaptic depression evoked in control slices is mediated by $GABA_B$ receptors. A conditioning train was applied to the "conditioning" pathway preceding every 10th stimulus delivered to the "test" pathway. A, An example of one experiment from a control slice. Heterosynaptic depression was observed as a reduction in amplitude of the fEPSPs that were preceded by a train (open circles) compared to fEPSPs not preceded by a train (each filled circle represents average of the nine unconditioned fEPSPs in each cycle). The opioid antagonist naloxone (10 μM) had no effect on the magnitude of depression. The $GABA_B$ receptor antagonist SCH50911 (20 μM) abolished the depression. fEPSPs (averages of 5 trials each) are shown in the absence and presence of SCH50911. B, Summary of the ratio of conditioned/unconditioned fEPSP amplitudes in 8 control slices, showing the abolition of heterosynaptic depression by blocking $GABA_B$ receptors.

Thus, in slices from control adult rats, heterosynaptic depression evoked with this protocol was entirely mediated by $GABA_B$ receptors (Min et al, 1998; Vogt and

Nicoll, 1999). To confirm that there was no contribution from metabotropic glutamate receptors, I applied SCH 50911 to one slice as in previous experiments, and then added the broad spectrum (group I and group II) (Eaton et al, 1993) metabotropic glutamate receptor antagonist (RS)-α-methyl-4-carboxyphenylglycine (MCPG) (500 μM). This is a concentration that was effective in reducing heterosynaptic depression in similar experiments by Min et al (1998). It had no significant effect on the fEPSP ratio, confirming that group I/II metabotropic glutamate receptors are not involved in this phenomenon (fEPSP ratio in naloxone only = 0.78, SCH 50911 = 0.93, SCH 50911 + MCPG = 0.90). This differs from the results in guinea-pigs observed by Min et al (1998) and Vogt and Nicoll (1999)

.

Heterosynaptic interactions mediated by spillover of glutamate are temperature dependent in that spillover is decreased at higher temperatures (Asztely et al, 1997). This may be due to the high Q_{10} of transporters, to a larger fraction of the glutamate transporters being exposed to the extracellular space as temperature increases, or to temperature-dependent modulation of transporter function. I asked if this was also true of GABA mediated depression. I measured heterosynaptic depression at 34°C, using the same stimulation protocol. $GABA_B$ receptor mediated heterosynaptic depression was identical to that recorded at room temperature (21 ± 8 % depression, n = 3 slices from different animals, p = 0.9, unpaired t test when compared to depression at room temperature, Fig. 3.9).

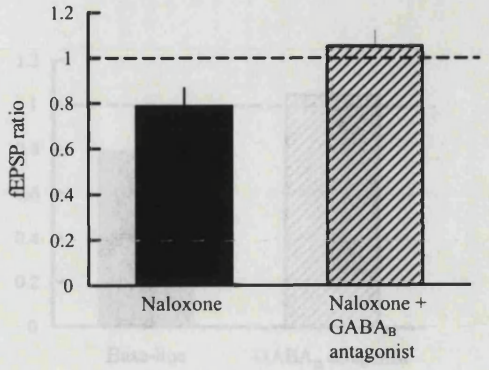


Figure 3.9: GABA$_B$ receptor mediated heterosynaptic depression is still present at physiological temperature (32-34°C), and of the same magnitude as that recorded at room temperature (see Fig. 3.8B).

Finally, I asked if there was any GABA$_B$ receptor mediated tonic inhibition. Blocking GABA$_B$ receptors caused a small increase in the size of the unconditioned fEPSPs in 2/8 control slices (Fig. 3.8A). There was no significant effect of the GABA$_B$ receptor antagonists on the unconditioned fEPSP when all results were averaged (base-line fEPSP amplitude = 0.21 ± 0.07 mV, fEPSP amplitude in presence of GABA$_B$ antagonist=0.22 ± 0.06 mV, $p = 0.45$ paired t test, $n = 8$). Hence, tonic GABA$_B$ receptor mediated inhibition was not a consistent finding in these experiments.

I performed all of the previous experiments in 4 mM Ca^{2+} and 4 mM Mg^{2+} to prevent any epileptiform bursting in CA3 (see Chapter 2: General Methods, "Electrophysiology"). To verify that the phenomenon of heterosynaptic depression is present at more physiological concentrations of divalent cations, I repeated the experiments with a perfusion solution containing 2.5 mM Ca^{2+} and 1.3 mM Mg^{2+}. In

two slices this yielded heterosynaptic depression of 33 ± 4 %, which was reduced to 4 ± 2 % with $GABA_B$ receptor antagonists.

Although $GABA_B$ receptors profoundly depress transmitter release, a potentially confounding effect is efflux of K^+ from neurons secondary to activation of G protein coupled potassium channels (Sodickson and Bean, 1996), which could alter axon recruitment and/or action potential propagation. An increase in extracellular potassium has previously been shown to decrease the presynaptic fibre volley at CA3-CA1 synapses (Frerking et al, 2001). I therefore compared the presynaptic fibre volleys (recorded in the presence of NBQX to abolish the fEPSP) with and without the 50 Hz train in the conditioning pathway. Neither the amplitude (fibre volley without train = 0.08 ± 0.04 mV, fibre volley in presence of train = 0.08 ± 0.04 mV; p = 0.69, paired t test, n = 8) nor the shape of the fibre volley was significantly affected (Fig. 3.10), implying that heterosynaptic depression is not mediated by an increase in extracellular K^+.

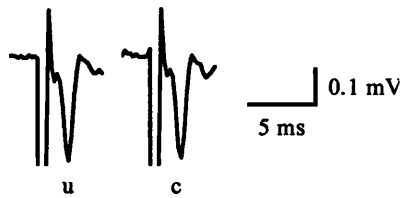


Figure 3.10: The fibre volley was unchanged in both shape and amplitude in the presence of the conditioning train. Example of presynaptic fibre volleys from one experiment showing that the conditioned presynaptic fibre volley (preceded by a conditioning train, c) is not different in amplitude or shape from the unconditioned fibre volley (not preceded by a train, u). Traces are averages of responses in the

presence of 50μM NBQX from two cycles of 9 unconditioned stimuli and one conditioned stimulus. The intensity of the stimuli was unchanged from that used to evoke heterosynaptic depression prior to addition of NBQX.

3.4 Discussion

In this study I have shown that $GABA_B$ receptor mediated heterosynaptic depression can be induced at the hippocampal mossy fibre synapse in slices taken from adult control rats.

In order to identify mossy fibre responses, I measured frequency facilitation and sensitivity to DCG-IV; the responses that I recorded showed a facilitation of at least 200% increase in amplitude and a decrease to at least 30% of baseline, respectively. I chose these criteria to define mossy fibre responses because others have shown that they are specific for mossy fibre synapses and have used them to confirm that responses are of mossy fibre origin. Kamiya et al (1996) showed that DCG-IV reduced the amplitude of putative mossy fibre responses but did not affect commissural/associational inputs onto CA3, or Schaffer collateral synapses between CA3 and CA1. They also showed that frequency facilitation was enhanced by DCG-IV and that responses evoked by pressure application of glutamate in *stratum lucidum* were not affected by it. These lines of evidence strongly support that DCG-IV acts on presynaptic receptors on mossy fibre terminals to reduce transmitter release at the mossy fibre synapse. Presynaptic group II metabotropic glutamate receptors also inhibit transmitter release at synapses between medial perforant path and dentate granule cells (Macek et al, 1996).The perforant path also projects directly to CA3 (Berzhanskaya et al, 1998). Contamination of my putative mossy

fibre responses by perforant path input will thus not be detected by sensitivity to DCG-IV. I therefore used frequency facilitation as an additional criterion to confirm that responses were of mossy fibre origin which was a mossy fibre characteristic reported by Salin et al (1996). This characteristic is unlikely to be exhibited by the perforant path since the lateral and medial perforant path exhibit short-term depression at dentate granule cell synapses (White et al, 1979; Kilbride et al, 2001; Rush et al, 2002) and have similar physiological properties at the CA3 synapses (Berzhanskaya et al, 1998)

I could elicit heterosynaptic depression in the adult rat hippocampus using the stimulation protocol described by Vogt and Nicoll (1999) and similar to that described by Min et al (1998). However in adult control rats I have shown that it is mediated solely by $GABA_B$ receptors without a contribution from metabotropic glutamate receptors. This contrasts with the phenomenon in guinea-pigs which did have a metabotropic glutamate receptor mediated component. Possible explanations for this are: different expression of metabotropic glutamate receptors on mossy fibre terminals in adult rats compared to juvenile guinea-pigs or less glutamate spillover, more glutamate uptake or reduced diffusion due to more tortuous extracellular space in this age and species.

Although Weisskopf et al (1993) have observed an opioid mediated heterosynaptic depression at the mossy fibre synapse, I showed that the opioid antagonist naloxone did not affect the magnitude of heterosynaptic depression. This can be explained by

the fact that they used a much higher frequency of stimulation to induce depression, which may be necessary to release opioids from mossy fibres (Wagner et al, 1990). Heterosynaptic depression is present in physiological conditions and does not appear to be due to an increase in extracellular potassium. How does activation of $GABA_B$ receptors cause heterosynaptic depression? G-protein-mediated signalling through presynaptic $GABA_B$ receptors can depress transmission in two ways. First, $GABA_B$ receptor activation reduces calcium influx (Takahashi et al, 1998; Isaacson and Hille, 1997) via inhibition of P/Q-(Barral et al, 2000; Mintz and Bean, 1993) and N-type (Easter and Spruce, 2002) calcium channels. This reduction in intraterminal calcium reduces vesicle recruitment. However, Scanziani et al (1992) showed that inhibition of presynaptic calcium currents was not necessary for inhibition of spontaneous action potential independent miniature excitatory synaptic currents by baclofen. This implies the existence of additional mechanisms linking $GABA_B$ receptors to exocytosis. In fact it has been recently shown that $GABA_B$ receptor agonists retard the recruitment of synaptic vesicles during sustained activity at the calyx of Held synapse (Sakaba and Neher, 2003). The retardation occurs through a lowering of cyclic AMP which blocks the stimulatory effect of increased calcium concentration on vesicle recruitment. One or a combination of these mechanisms can explain how $GABA_B$ receptors mediate the depression of transmission at mossy fibre synapses that I observed here.

$GABA_B$ receptors are present both pre- and postsynaptically at the mossy fibre synapse. Heterosynaptic depression has previously been attributed to presynaptic

receptors only. Could post-synaptic $GABA_B$ receptors also play a role? Takigawa and Alzheimer (2002) have shown that the $GABA_B$ receptor agonist baclofen attenuates EPSPs recorded at the soma by whole cell patch-clamp in cultured CA1 neurons by approximately one-third via activation of G-protein-activated inwardly rectifying potassium channels (GIRKs) on post-synaptic cells. It is therefore possible that post-synaptic $GABA_B$ receptors could play a role. However this is difficult to put into the context of my study because I was recording extracellular fEPSPs in the dendrites of CA3 pyramidal neurons.

I also demonstrated that the phenomenon was unaffected by temperature. This is in contrast to glutamate spillover phenomena (Asztely et al, 1997). Why should this be the case? Glutamate transporters are less active at lower temperatures (Wadiche and Kavanaugh, 1998; Diamond and Jahr, 2000). This may not necessarily be the case for GABA transporters in this region of the hippocampus although there is some evidence that GABA uptake is increased at higher temperatures (Binda et al, 2002). If however GABA transporter function is upregulated at higher temperatures, my results could be explained by greater GABA release at higher temperatures. This is in keeping with the finding that tonic $GABA_A$ receptor activation is unaffected by temperature (Semyanov et al. 2003).

Since modulatory phenomena at the mossy fibre synapse can potentially gate limbic seizure propagation, I studied this phenomenon in rodent seizure models to determine whether a change in heterosynaptic depression could play a role in

epilepsy. An additional question is whether the GABA that causes heterosynaptic depression is released from mossy fibres or interneurons. This question is addressed in Chapters 4 and 5 respectively.

Chapter 4: Is heterosynaptic depression altered after status epilepticus?

4.1 Introduction

Most naturally occurring seizures only last a maximum of a few minutes but occasionally seizure activity develops into a self-sustaining seizure state, which is termed status epilepticus (Hopkins et al, 1995). The intrinsic mechanisms that stop most naturally occurring seizures and the changes that allow status epilepticus to develop are not understood (Dragunow, 1986; Young and Dragunow, 1994). Following a period of status epilepticus, both humans and animals frequently develop spontaneous seizures (Leite et al, 2002; Herman, 2002). There are several changes including axonal reorganisation (Represa et al, 1990) and altered receptor expression that occur during the latent period between status epilepticus and the emergence of spontaneous seizures (see Chapter 1: General Introduction). Could the phenomenon that I described in the previous chapter, heterosynaptic depression, be involved in these phenomena; the development of status epilepticus, and the later emergence of spontaneous seizures? I first asked if heterosynaptic depression was changed following experimentally induced status epilepticus.

4.2 Methods

Acute hippocampal slices were prepared from adult rats 24 hours after 90-120 minutes of status epilepticus induced by pilocarpine administration or perforant path stimulation (see Chapter 2: General Methods). Mossy fibre fEPSPs were identified

using identical methods to those used in the previous chapter. The same two pathway stimulation protocol was used to study heterosynaptic depression as previously described.

4.3 Results

I identified two mossy fibre pathways in slices from animals after pilocarpine-induced status epilepticus using the same protocol as in chapter 4.

To determine if mossy fibre responses had the same properties in control and post-status epilepticus slices, I compared the degree of frequency facilitation and sensitivity to DCG-IV in controls and post-status epilepticus slices. Frequency-dependent facilitation was not significantly different from that measured in control slices (post-SE: 232 ± 25 % of baseline, n = 12; control: 358 ± 67 %, n = 8; measured as 20th fEPSP / 1st fEPSP amplitude when stimulation was increased from 0.05Hz to 1Hz, p = 0.1, Fig. 5.1).

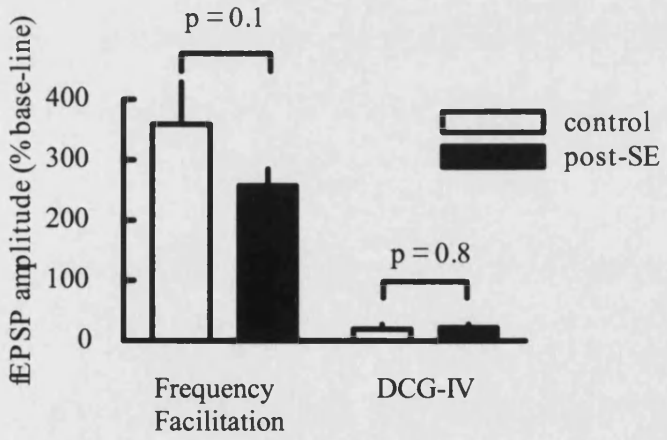


Figure 4.1: Mossy fibre fEPSPs in slices after pilocarpine-induced SE (n = 12) showed frequency facilitation and DCG-IV sensitivity which were not significantly different from that observed in control slices.

It has been well described that sprouting of mossy fibre axons into the inner molecular layer occurs following status epilepticus (Okazaki et al, 1995; Buckmaster et al, 2002; Zhang and Houser, 1999) and this could potentially have made identification of separate mossy fibre pathways difficult. However, this was not a problem because these slices were taken 24 hours following status epilepticus. Sprouting takes several days to develop (Mello et al, 1993). I used the same criteria to confirm that the two pathways were separate (cross-facilitation = 0 ± 6 % n = 11). Moreover, in post-SE slices, DCG-IV perfusion reduced the fEPSPs to 22 ± 5 % of baseline. This depression was also indistinguishable from that seen in control slices (19 ± 8 %, p = 0.8, Fig. 4.1). I then used the same protocol as used in chapter 4 to stimulate the two pathways in order to induce heterosynaptic depression. However, in contrast to control animals, heterosynaptic depression was not detected after pilocarpine induced SE (1 ± 4 % depression, n = 12, p = 0.75, paired t test comparing conditioned to unconditioned fEPSPs, Fig. 4.2).

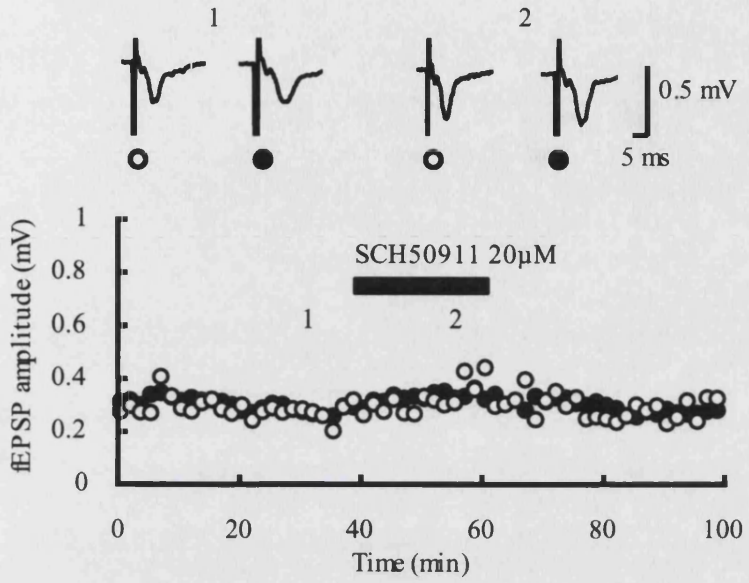


Figure 4.2: $GABA_B$ receptor mediated heterosynaptic depression is absent after SE. Heterosynaptic depression was absent after pilocarpine-induced SE: example of one experiment. fEPSPs are shown in the absence and presence of SCH50911. (Each trace is the average of 5 trials; conditioned: filled circles, unconditioned: open circles). The $GABA_B$ receptor antagonist SCH50911 had no significant effect.

Thus in slices from SE experienced animals, in spite of the apparently normal short-term plasticity and metabotropic glutamate receptor sensitivity of mossy fibre synapses, they were unaffected by trains of stimuli delivered to neighbouring axons.

These results do not distinguish between a generic effect of limbic seizures and a specific result of the chemoconvulsant used to evoke SE. I therefore asked whether a different model of SE, which does not involve the use of an exogenous convulsant, also interfered with $GABA_B$ receptor-mediated modulation of mossy fibre transmission. I examined hippocampal slices taken from six animals 24 hours after

SE evoked by perforant path stimulation (see Chapter 2: General Methods). Heterosynaptic depression was again absent in slices from all six animals (-2 ± 5 % depression, n = 6, Fig. 4.3A).

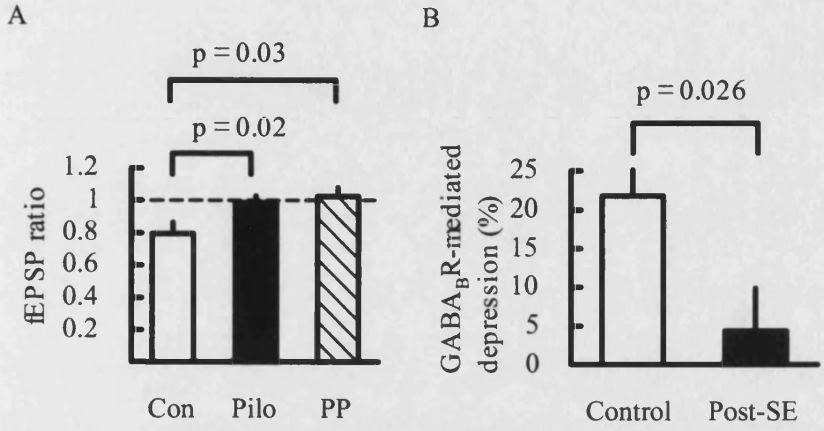


Figure 4.3: A There was no significant heterosynaptic depression in animals after pilocarpine-induced or perforant path stimulation-induced SE in contrast to the marked depression seen in control animals. B Size of $GABA_B$ receptor mediated heterosynaptic depression, estimated from the effect of the $GABA_B$ antagonists SCH50911 or CGP52432 on the fEPSP ratio. SE: both models combined (n = 18).

I also examined slices from two animals treated with pilocarpine that had not developed SE. Both animals received diazepam injections identical to those administered to the animals that experienced SE. Heterosynaptic depression was observed in slices from these animals (45 ± 4 % depression). Thus, the loss of heterosynaptic depression is related to SE *per se*, rather than to the stimulus used to evoke it.

To test whether the loss of heterosynaptic depression post-SE was due to the emergence of a compensatory heterosynaptic facilitation, I applied the $GABA_B$ receptor antagonists SCH50911 (20 μM) or CGP52432 (5μM) during the two pathway protocol in slices post-SE. This had no significant effect on the fEPSP ratio in slices from the post-SE animals (5 ± 4 % change in the fEPSP ratio, n = 18, Fig. 4.3B). This was significantly different from controls (22 ± 3 % change in the fEPSP ratio, n = 8) when $GABA_B$ receptor antagonists were applied (p = 0.026 unpaired t test for difference between SE and control animals). This result implies that loss of heterosynaptic depression post-SE was not due to the emergence of a compensatory heterosynaptic facilitation, but because of failure of $GABA_B$ receptor-mediated depression.

4.4 Discussion

I have demonstrated that $GABA_B$ receptor-mediated heterosynaptic depression at the rat mossy fibre synapse is lost after SE. This is observed in animals after both pilocarpine and perforant path stimulation induced status epilepticus. This loss is not due to a compensatory heterosynaptic facilitation, as shown by the application of $GABA_B$ antagonists. The mossy fibre responses in the epileptic animals had frequency facilitation and sensitivity to DCG-IV that were not significantly different from controls.

The non-significant trend for less frequency facilitation in the post-SE animals is consistent with the findings of Goussakov et al (2000), who showed a significant

loss of facilitation after kainic acid induced SE. The more marked changes observed by Goussakov et al. (2000) may be due to differences in (i) the method used to induce SE (kainic acid vs. pilocarpine), (ii) stimulation frequency (100 Hz instead of 1 Hz) and/or (iii) a longer interval from SE to sacrifice of the animals (several weeks instead of 24 hours). I selected mossy fibre responses on the basis of their frequency facilitation and DCG-IV sensitivity. I accepted fEPSPs that showed a 200 % increase in amplitude. This means that responses were rejected if they did not show sufficient facilitation and could have led to a skew in the selection process. If I rejected some responses that were indeed mossy fibres but had less facilitation this might also explain the discrepancy between mine and the results of Goussakov (2000).

This loss of a $GABA_B$ receptor mediated phenomenon could have important implications for the use of antiepileptic drugs that act via $GABA_B$ receptors. Although there are no $GABA_B$ receptor agonists currently used as antiepileptic drugs, several *in vitro* studies have shown that they have antiepileptic properties (Ault et al, 1986; Swartzwelder, 1986a,b). Does the downregulation of a $GABA_B$ receptor mediated phenomenon affect the efficacy of $GABA_B$ receptor agonists in these models? I address this question in later chapters. Why else could the loss of $GABA_B$ receptor mediated heterosynaptic depression be important? First it could be a mechanism by which self-sustaining status epilepticus develops. Second it could be one phenomenon that increases hippocampal excitability and allows spontaneous seizures to occur.

Two important questions arise from this finding. First, from where is the GABA that mediates heterosynaptic depression released? Is it from the mossy fibres themselves, or interneurons that are recruited by mossy fibres? Second, what mechanisms lead to loss of heterosynaptic depression after status epilepticus? This could be due to reduced GABA release from mossy fibres or inhibitory interneurons, loss of GABAergic cells or reduced recruitment of GABAergic cells, increased GABA uptake, altered extracellular space or loss of functional $GABA_B$ receptors. These questions will be addressed in the following chapters.

Chapter 5: Is the GABA that causes heterosynaptic depression in control slices released from interneurons or mossy fibres?

5.1 Introduction

The stimulation protocol that I used to induce heterosynaptic depression consisted of a train of stimuli applied to one mossy fibre pathway which inhibited transmission in an adjacent mossy fibre pathway. In previous experiments I have shown that this depression of transmission is mediated via $GABA_B$ receptors. Which cell types release the GABA that mediates this heterosynaptic depression? Vogt and Nicoll (1999) did not directly address the question of the source of GABA experimentally but they suggested that the main source was local inhibitory interneurons and that perhaps mossy fibres also contributed to the GABA release. Could mossy fibres be the source of GABA for heterosynaptic depression? This is paradoxical because mossy fibres are usually considered to form excitatory synapses. They contain high concentrations of glutamate (Storm-Mathisen et al, 1983; Bramham et al, 1990) express the vesicular glutamate transporter VGLUT1 (Fremeau et al, 2001) and release glutamate at their synapses onto CA3 pyramidal neurons (Jonas et al, 1993). However, as well as containing glutamate, mossy fibres also contain the GABA synthesizing enzyme GAD67 (Dupuy et al, 1996; Schwarzer and Sperk, 1995a) and GABA itself (Sloviter et al, 1996; Woodson et al, 1989; Taupin et al, 1994). In addition, recent reports have shown that a GABAergic response with mossy fibre-like properties can be recorded in CA3 pyramidal neurons when mossy fibres are

stimulated (Walker et al, 2001; Bergersen et al, 2003;Gutierrez, 2000;Gutierrez and Heinemann, 2001). When electrical or chemical stimuli designed to recruit mossy fibres were applied to a slice, monosynaptic GABAergic responses were recorded in CA3 pyramidal neurons which remained when glutamate receptors were blocked (Walker et al, 2001). This $GABA_A$ receptor mediated response exhibits the same frequency facilitation, NMDA receptor independent long-term potentiation and metabotropic glutamate receptor agonist sensitivity as the glutamatergic component, providing compelling albeit circumstantial evidence that mossy fibres do indeed release GABA. Gutierrez (2000) and Gutierrez and Heinemann (2001) reported that following seizures, a GABAergic potential can be recorded in CA3 pyramidal neurons when mossy fibres are stimulated, that is not present in control slices. A similar phenomenon was observed in juvenile rat tissue (Gutierrez et al, 2003). These studies strongly suggest that mossy fibres release GABA and that the GABAergic component of the postsynaptic component increases after seizures. It is therefore possible that stimulation of the conditioning pathway in my protocol induces GABA release from mossy fibres. If this spills over to adjacent synapses it could induce $GABA_B$ receptor mediated heterosynaptic depression.

An alternative possible source of GABA is interneurons recruited by mossy fibres. Although the stimulating electrode that delivered the train of 5 stimuli to the conditioning pathway was positioned in the dentate granule cell layer where it recruited mossy fibres, these in turn may recruit other cells. Indeed, one dentate granule cell provides input to only 11-18 CA3 pyramidal neurons (Acsady et al,

1998) and ~150 hilar cells (Henze et al, 2000), most of which are inhibitory interneurons. However, direct stimulation of interneurons projecting to CA3 is likely to be minimal with a stimulating electrode positioned in *stratum granulosum* (Walker et al, 2001). Since mossy fibres make many more synapses with inhibitory interneurons than with CA3 pyramidal neurons, the stimulation of mossy fibres is likely to recruit large numbers of interneurons. There are many interneuronal subtypes in the hippocampus (Freund and Buzsaki, 1996). Which of these are most likely to be involved in release of GABA that mediates heterosynaptic depression? Mossy fibres provide a robust innervation to all types of interneurons that have dendrites in *stratum lucidum* (Henze et al, 2000) and to basket cells in CA3 (Frotscher, 1985). They also make connections with mossy fibre-associated interneurons in *stratum lucidum* which then synapse onto the proximal dendritic shafts and somata of pyramidal neurons and onto inhibitory interneurons (Vida and Frotscher, 2000). Since these mossy-fibre associated interneurons are GABAergic cells that reside in *stratum lucidum* they could contribute to the GABA that mediates heterosynaptic depression. However, none of these interneurons are known to form GABAergic synapses in *stratum lucidum.*

If mossy fibres release the GABA that mediates heterosynaptic depression, why is the depression lost following status epilepticus? One possible explanation is that GABA is depleted in mossy fibres following prolonged seizures. However, an *increase* in GABA has been reported after prolonged perforant path stimulation (Sloviter et al, 1996) and kindling (Lehmann et al, 1996; Gomez-Lira et al, 2002)

and the GABA synthesizing enzyme GAD67 is increased after kainic acid induced seizures (Schwarzer and Sperk, 1995). I asked if GABA was depleted in mossy fibres after status epilepticus induced by pilocarpine by using immunogold labelling of GABA. This method was used so that I could confirm that the GABA measured was within the mossy fibre terminals themselves and not in other surrounding GABAergic cells.

If, alternatively, the GABA is released from interneurons, then preventing GABA release from interneurons should abolish depression. Endogenous cannabinoids can depress GABA release from specific interneurons during a process known as depolarization-induced suppression of inhibition (DSI) (Wilson and Nicoll, 2001). When hippocampal pyramidal neurons are depolarized they release the endogenous cannabinoids anandamide and 2-arachidonylglycerol which act at the G protein-coupled cannabinoid receptor-1 (CB1). They transiently suppress GABA-mediated transmission via these receptors. Since endocannabinoids reduce GABA release from interneurons, I asked if application of an endocannabinoid agonist reduced heterosynaptic depression in control slices.

Finally, if the GABA is released from mossy fibres, I hypothesized that increasing the release probability in the conditioning train will increase mossy fibre GABA release and hence, an increase in the magnitude of heterosynaptic depression. I did this by inducing long-term potentiation (LTP) in the conditioning train. Mossy fibres exhibit an unusual form of LTP distinct from that seen at other synapses in that it is

independent of NMDA receptor activation (Harris and Cotman, 1986; Zalutsky and Nicoll, 1990). This is in contrast to the NMDA receptor dependent form seen at the Schaffer collateral synapse in CA1 (Bliss and Collingridge, 1993). I used this property to induce LTP in a mossy fibre pathway while blocking NMDA receptors, thus preventing LTP induction at commissural-associational synapses. This protocol should not induce LTP at mossy fibre – interneuron synapses because mossy fibre – interneuron synapses exhibit long-term depression or no change when stimulated at high frequency (Toth et al, 2000; although see Alle (2001). However, it may result in an increase in GABA release from mossy fibre synapses onto CA3 pyramidal cells (Walker et al, 2001). I asked if increasing release probability by inducing LTP in the conditioning mossy fibre pathway increases the magnitude of depression.

5.2 Methods

5.2.1 Immunogold labelling for GABA

The GABA immunogold experiments were carried out by Dr. Ruth Fabian-Fine of Dalhousie University, Canada. I prepared the pilocarpine treated animals (see General Methods) and she performed the electron microscopic studies. For electron microscopy (EM) postembedding immunogold labelling, brain sections were obtained from adult male Sprague-Dawley rats (2 controls and 2 post-status epilepticus, induced by pilocarpine that were killed 24 hours after status epilepticus). The rats were perfused over 20 minutes with 4% paraformaldehyde while under deep pentobarbitone anaesthesia. Ultrathin sections (50 nm) were cut with a Reichert Ultracut and collected on pioloform-coated single-slot nickel grids. Grids

were then mounted in a grid support plate, soaked in phosphate buffer (PB) for 30 min and preincubated in an incubation medium (IM) consisting of PB with 1% bovine serum albumin (A4503, Sigma) and 5% foetal calf serum for 30 min at room temperature. Sections were then incubated with a rabbit anti-GABA antibody (1:4000 in IM; A2052; Sigma) overnight at 4°C. After thorough washing (4x10 min in PB) and preincubation in IM (30 min), the secondary antibody (goat anti-rabbit IgG coupled to 10 nm gold particles; Sigma) was applied at a dilution of 1:100 in IM for 4 h at 37°C. Preparations were washed subsequently in PB (5x10 min) before final rinsing in double-distilled water. The sections were contrasted with uranyl acetate (4 min) and Reynold's lead citrate (50 sec) according to standard EM methods. Preparations were examined using a Philips 201C electron microscope. Control preparation from which the primary antibody was omitted showed no immunolabelling.

Mossy fibres contain high concentrations of zinc. This property is used to label mossy fibres at light microscopic level using a stain for zinc developed by Timm (1958). In this method sodium sulphide is used to precipitate any zinc contained in the mossy fibres. Metallic silver is then used to enlarge the resulting zinc sulphide grains by means of the Liesegang's physical developer containing gum arabic as protective colloid. This method is not suitable for labelling zinc when examining at the ultrastructural level, because the silver grains are large and the ultrastructure is not sufficiently preserved. The Timm's stain was therefore modified by Seress and Gallyas (2000) to make it suitable for use at electron microscopic level. This method

uses sodium tungstate as a protective colloid. This developer ensures that the ultrastructure is preserved and that small silver grains are produced. The modified Timm's stain was used in this study to confirm the identity of mossy fibre terminals. This method was used in two additional animals: one control and one post-SE. Since a different fixation method was used for these two animals, they were not included in the quantitative analysis. The animals were perfused over 1 min with a buffered Na_2S solution followed by 3% glutaraldehyde in 0.1% phosphate-buffered saline (PBS, pH 7.4, 20 min) and again with Na_2S solution (15 min). After dissection, brains were postfixed in 3% glutaraldehyde for 2 h on ice. Vibratome sections (50 µm) were washed in Tris buffer (pH) 7.4 and placed in tungstate physical developer (pH 5.5, 15 min) at room temperature. The reaction was stopped by placing the sections into 1% sodium thiosulphate (1 min). After final rinsing in Tris buffer, sections were osmicated (0.5% for 15 min), dehydrated, and embedded in Araldite according to standard protocols. For EM immunogold labeling, ultrathin sections (50 nm) were collected on pioloform-coated single-slot nickel grids. Grids were then mounted in a grid support plate and etched for 25 min in 1% periodic acid in distilled water. After washing in distilled water (3 x 5 min) grids were exposed to 2% sodium metaperiodate (25 min), rinsed in distilled water, and preincubated in incubation medium (IM) consisting of 1% bovine serum albumin and 10% fetal calf serum in PBS (30 min). Sections were then incubated in the primary and then secondary antibody as described for the previous animals.

5.2.3 Effect of WIN 55,212-22 on heterosynaptic depression

I compared the magnitude of heterosynaptic depression in the absence and presence of WIN 55,212-22 (800nM, dissolved in DMSO) while recording two pathway experiments in control slices.

5.2.2 Induction of long-term potentiation

LTP was induced by applying 3 trains of 100 pulses at 100 Hz with an inter-train interval of 10s. LTP was defined as an increase in fEPSP amplitude to at least 150% of base-line at 10 minutes after induction, persisting to the end of the experiment. Slices were rejected if this was not achieved. Heterosynaptic depression was measured 10 minutes following induction of LTP. LTP was induced in the presence of the NMDA receptor antagonist D-APV (50 μM) to ensure that only mossy fibres were potentiated.

5.3 Results

Immunogold electron microscopy was used to detect GABA-like immunoreactivity in mossy fibre terminals, in control tissue and tissue from rats 24 hours after pilocarpine induced status epilepticus. Figure 5.1 shows examples of electron micrographs taken from *stratum lucidum*. Mossy fibre terminals were identified by their large size, multiple active zones and numerous vesicle profiles. In addition, the modified Timm's reaction was used in one control and one post-SE animal to confirm the identity of the mossy fibre terminals on the basis of their high zinc content (Seress and Gallyas, 2000). I did not use the Timm's stained preparations for quantitative analysis because of the different fixation method used. Immunogold particles in mossy terminals of control animals (Figure 5.1C,D) were present at a density of 12 ± 1 per μm^2 (56 terminals from two animals). This was significantly greater than the background density of 4 ± 1 per μm^2 ($p < 0.001$). The measurements were repeated in 2 post-SE animals (Fig. 5.1A,B). The GABA immunogold particle

density in mossy fibre terminals was *doubled* 24 hours after SE induced by pilocarpine to 24 ± 2 per μm^2 (45 terminals from two animals, $p < 0.001$ compared to controls). Thus, the immunolabelling, while confirming more indirect evidence for GABA in mossy fibres, lends no support to the hypothesis that the amount available to be released is decreased following SE. On the contrary, there is more GABA in mossy fibres following SE, in keeping with previous reports that seizure-like activity is followed by the emergence of an upregulation of GABA in mossy fibres (Sloviter et al, 1996) and a monosynaptic GABAergic signal in CA3 pyramidal neurons (Gutierrez and Heinemann, 2001).

Cannabinoid agonists reduce GABA release from interneurons via activation of CB1 receptors (Wilson and Nicoll, 2001). I applied the endocannabinoid agonist WIN 55,212-22 (800nM) while recording two pathway experiments in control slices. This concentration was used because Wilson and Nicoll (2001) showed that this was sufficient to depress IPSC amplitudes in slice preparations. The heterosynaptic depression did not significantly change in the presence of WIN55,212-22 when compared to depression in base-line conditions ($n = 3$, $p = 0.49$, paired t test, Fig. 5.2). In these three experiments the mean fEPSP ratio in the presence of naloxone only, was 0.80 ± 0.08. This was abolished (0.0 ± 0.1 % depression) by the $GABA_B$ receptor antagonist.

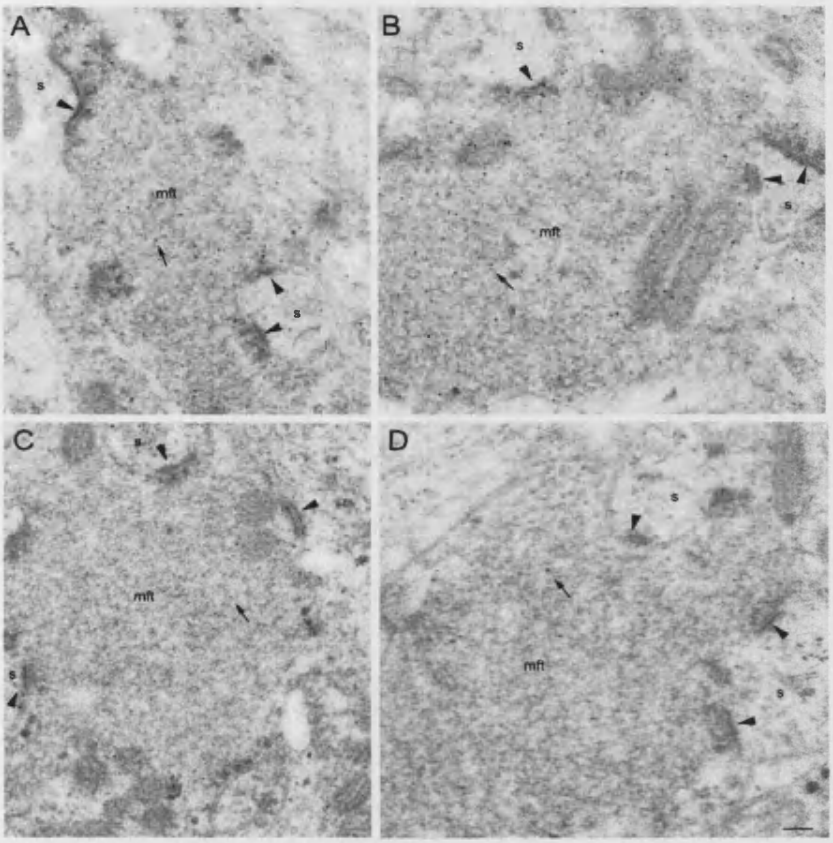


Figure 5.1: Sections through GABA immunogold-labelled mossy fibre terminals in SE (A, B) and control (C, D) animals. Tissue from animals after SE have significantly higher density of immunogold particles (arrows). Arrowheads, synaptic sites; mft, mossy fibre terminal; s, spine. Scale bar: 100 nm (A,B), 120 nm (C), 60nm (D).

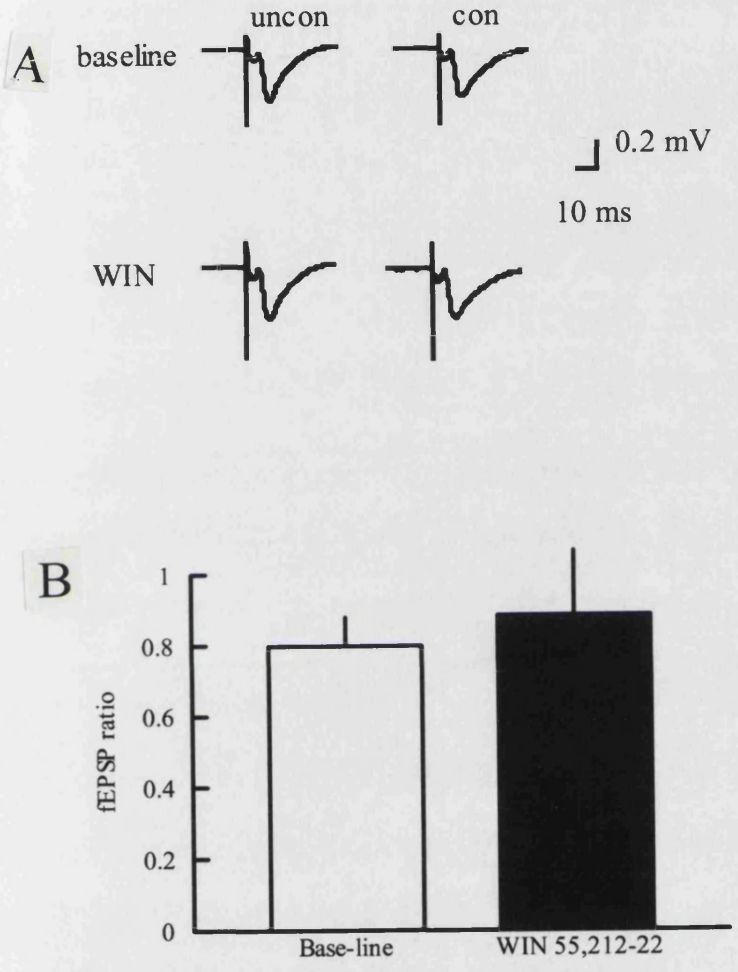


Figure 5.2: The endocannabinoid agonist WIN 55,212-22 did not significantly change the magnitude of heterosynaptic depression in control slices (baseline fEPSP ratio = 0.80 ± 0.08, WIN fEPSP ratio = 0.88 ± 0.18).(con = conditioned, uncon = unconditioned).

I then asked if induction of LTP in the conditioning mossy fibre pathway leads to increased heterosynaptic depression. If the GABA that mediates heterosynaptic depression is released from mossy fibres I would expect to see an increase in depression after the induction of LTP in the mossy fibre conditioning train, because this should increase mossy fibre GABA release. In the presence of D-APV (50 μM), I induced LTP in the conditioning train in 5 slices from control animals. I used 3 trains of 100 Hz stimuli, of duration 1 s, which were applied every 10 seconds (Urban and Barrionuevo, 1996). Slices were rejected if the increase in amplitude was to less than 150 % of base-line (measured 10 minutes after applying the trains) or if the increase was not persistent throughout the experiment. The mean amplitude after induction was 248 ± 43 % of base-line at 10 minutes after induction (n = 5) (Fig. 5.3).

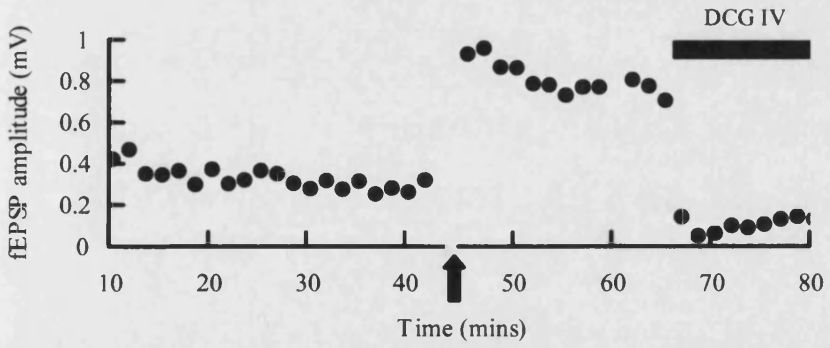


Figure 5.3: I applied 3 trains of 100 pulses at 100 Hz at 10 s intervals (arrow) to induce LTP in the conditioning train. This graph demonstrates the amplitude of the first fEPSP in each 50 Hz conditioning train before and after LTP induction. In this experiment the amplitude increased to 265 % of base-line when measured 10 minutes after LTP induction. DCG-IV(1 μM) was applied at the end of the experiment to confirm that responses were mossy fibre in origin.

I applied the two pathway protocol both before and after the induction of LTP. I compared the fEPSP ratio under base-line conditions and ten minutes after LTP induction. After LTP induction there was a trend for increased depression but it did not reach significance. The pre-LTP fEPSP ratio was 0.59 ± 0.05 and the post-LTP fEPSP ratio was 0.48 ± 0.05 (Fig. 5.4, n = 5, p = 0.15 paired t test).

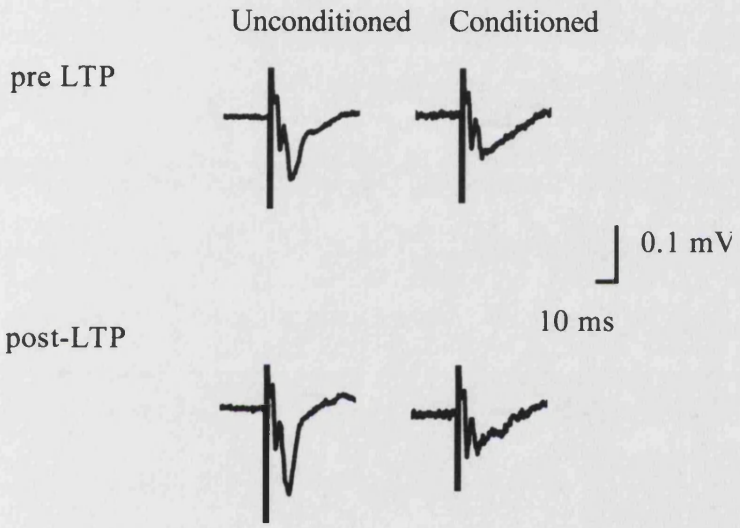


Figure 5.4: The fEPSP ratio during the two pathway protocol was measured before and ten minutes after the induction of LTP in the conditioning train. Although there was a trend towards a lower fEPSP ratio, the post-LTP ratio was not significantly different from the pre-LTP ratio. Heterosynaptic depression was therefore not significantly increased after induction of LTP in the conditioning train.

5.4 Discussion

In this chapter I explored the question: what is the source of GABA that mediates heterosynaptic depression? First, we demonstrated that there is an increase rather than a decrease in GABA concentration in mossy fibres after status epilepticus which means that they are unlikely to be the source of GABA. However I failed to abolish depression by the application of endocannabinoids, and was unable to increase significantly depression by increasing release probability in the conditioning train. No hard conclusions can be drawn from these data, and I have been unable to demonstrate a definite role for interneurons in the phenomenon of heterosynaptic depression.

The electron microscopic data are consistent with the work of others. Sloviter et al (1996) showed that immunolabelling for GABA and glutamic acid decarboxylase increase after seizure-like activity. This is accompanied by the emergence of a monosynaptic GABAergic signal (Gutierrez and Heinemann, 2001). Sloviter et al (1996) also found that GAD67-like immunoreactivity was present in the mossy fibres of rats, mice, and the monkey *Macaca nemestrina*. Perforant path stimulation for 24 hours, which evoked population spikes and epileptiform discharges, induced GAD65-, GAD67-, and GABA-like immunoreactivity in granule cells and pre-embedding immunocytochemical electron microscopy confirmed that GAD67- and GABA-like immunoreactivity were induced selectively within granule cells. I used a much shorter period of seizure activity in my models. I therefore wanted to confirm that there was an increase in GABA in the models that I was using and that the

increase is specifically in mossy fibres. The possibility cannot be excluded however, that mossy fibres are not able to release GABA following SE, perhaps due to a failure in the release mechanisms. This might lead to a build-up of GABA in the terminals. This seems unlikely however in the light of the findings of others who observed that a GABAergic signal emerges after seizures which was not present in controls (Gutierrez, 2000). In fact, this suggests the opposite, that GABA release from mossy fibres is *increased* after seizures.

I also explored the possibility that it is the interneurons that release the GABA that mediates heterosynaptic depression. I hypothesized that if it is indeed released from interneurons, depression will be reduced in the presence of endocannabinoid agonists, because activation of CB1 receptors inhibits the release of GABA from interneurons (Wilson and Nicoll, 2001). I however did not observe a significant change in the magnitude of depression in the presence of endocannabinoid agonists, although the small numbers mean that I cannot form any hard conclusions from these experiments. Why might a cannabinoid agonist not abolish depression; could this suggest that the source of GABA is not interneurons? There are a number of explanations for this result, even if the source of GABA *is* interneurons. First, 800 nM might not be sufficient to prevent GABA release. This seems unlikely because Wilson and Nicoll (2001) showed that this concentration was sufficient to decrease evoked IPSCs in slices. Second, is it possible that the interneurons responsible for heterosynaptic depression do not express CB1 receptors? CB1 receptors are widely expressed throughout the brain (Wilson and Nicoll, 2001). However, they are only

expressed on certain subtypes of interneuron. In the hippocampus CB1 is specific to cholecystokinin positive basket cells which synapse onto the somata of principal cells. There is an absence of CB1 immunostaining in *stratum lucidum* (Katona et al, 1999). It is therefore possible that CB1 agonists will not reduce GABA release from those interneurons that mediate heterosynaptic depression, for example the mossy fibre-associated interneurons which I described at the beginning of this chapter. Also, as can be seen in Fig. 5.2, the standard error increased in the presence of WIN, and there was a trend towards less depression suggesting that increasing the number of experiments in the presence of WIN 55,212-22 might reveal an effect on the magnitude of depression.

The final hypothesis was: if GABA is released from mossy fibres, increasing the release probability in the terminals within the conditioning train will increase depression. I did not observe a significant increase in depression following induction of LTP in the conditioning train which lends no support to the hypothesis that mossy fibres are the source of GABA. It should be noted though, that there was a non-significant trend towards more depression following LTP. This non-significant result could be due to an insufficient number of experiments, especially if there is a non-linear correlation between the magnitude of LTP and the increase in GABA detected by $GABA_B$ receptors (i.e. large LTP results in only a small increase in GABA concentration in the extracellular space). Are there other possible explanations for a trend towards increased heterosynaptic depression following LTP? Although the LTP experiments were done in the presence of NMDA receptor antagonists which

will prevent NMDA-dependent LTP being induced elsewhere in the hippocampus, the potentiated mossy fibres could recruit more CA3 pyramidal cells which in turn could recruit more interneurons as a form of feed back inhibition and hence increase depression. Also there have been mixed opinions as to whether LTP occurs at synapses between mossy fibres and interneurons. Some studies have shown that either no LTP or depression occurs (Maccaferri et al, 1998), and other more recent studies suggest that LTP can be induced (Alle et al, 2001).

The increase in mossy fibre GABA labelling following status epilepticus despite a reduction in heterosynaptic depression, and the lack of effect of mossy fibre LTP on heterosynaptic depression (despite evidence that this should increase GABA release from mossy fibres) provide indirect evidence that mossy fibres are not the main source of GABA mediating heterosynaptic depression. The failure of cannabinoids to decrease depression can be explained by an insufficient role of CB1-expressing interneurons in mediating heterosynaptic depression. Within the caveats discussed above (e.g. insufficient power to detect a difference, due to low n) my results suggest that the source of the GABA that mediates heterosynaptic depression is from interneurons and not mossy fibres. Further work is however necessary to provide more definitive evidence (see Chapter 9:General Discussion).

Chapter 6: Is loss of heterosynaptic depression due to a reduction in interneuron recruitment or a change in GABA uptake?

6.1 Introduction

In chapter 5, I demonstrated that $GABA_B$ receptor mediated heterosynaptic depression is lost after status epilepticus in two different epilepsy models. The source of the GABA that mediates the depression is not clear but could either be from local interneurons that are recruited by the conditioning train, or, less likely, from mossy fibres themselves.

Depending on the source of GABA, there are several possible explanations for the loss of heterosynaptic depression following status epilepticus. I will be addressing these questions over the following chapters. If GABA is released from interneurons, loss of depression could be due to reduced interneuron recruitment, a reduced number of interneurons or reduced GABA release from interneurons. If the GABA is released from mossy fibres, then loss of heterosynaptic depression could be due to a reduced GABA concentration in mossy fibres. However, the results described in Chapter 5 argue against this hypothesis. Regardless of the source of GABA, increased GABA uptake, decreased functional $GABA_B$ receptors or alterations in the extracellular space, could also lead to a loss of heterosynaptic depression.

First, if the source of GABA is interneurons, inhibitory interneurons could be lost or recruited less efficiently. Both human patients with temporal lobe epilepsy (de Lanerolle et al, 1989; Mathern et al, 1995) and animals after experimentally induced status epilepticus (Sperk et al, 1992; Sloviter, 1983) have fewer hippocampal interneurons than controls. Several studies have also demonstrated a reduced recruitment of interneurons following status epilepticus. It has been suggested that remaining interneurons are not recruited because of loss of vulnerable excitatory mossy cells which would usually recruit the relatively well preserved basket cell interneuron (the dormant basket cell hypothesis) (Bekenstein and Lothman, 1993; Sloviter et al, 2003; Sloviter, 1991; Jefferys and Traub, 1998). An upregulation of group II metabotropic glutamate receptors leading to a reduced excitatory drive and hence reduced recruitment of hilar border interneurons has also been described (Doherty and Dingledine, 2001). Second, GABA uptake could be increased following status epilepticus. This could abolish depression regardless of the source of GABA. Furthermore, altered GABA transporter function and expression have been reported during epileptogenesis. Patrylo et al (2001) reported a downregulation of GABA uptake after kainate induced status epilepticus and in patients with temporal lobe epilepsy. Drugs such as tiagabine, that inhibit the neuronal GABA transporter GAT1 (Andersen et al, 1993), are antiepileptic. A decrease in immunostaining for GAT1 in rodent epilepsy models has been reported in the sensorimotor cortex (Silva et al, 2002) and the hippocampus (Andre et al, 2001). Third, there could be reduced amount of GABA available to be released from mossy fibres and/or interneurons. However this hypothesis is difficult to reconcile with the

reports that show an increase in GABA concentration in mossy fibres after status epilepticus (Sloviter et al, 1996; Sandler and Smith, 1991) and emergence of a GABAergic current at the mossy fibre-CA3 synapse following seizures (Gutierrez, 2000; Gutierrez and Heinemann, 2001).

Fourth, $GABA_B$ receptor mediated heterosynaptic depression could be lost due to reduced expression of functional $GABA_B$ receptors. Again this could explain the loss of depression regardless of the source of GABA.

Lastly, alterations in the tortuosity of the extracellular space could provide an obstacle between the GABA release sites and the target receptors. Do changes in the extracellular space occur in epilepsy? Status epilepticus induces CNS oedema which may last several days (Roch et al, 2002). Cell swelling during excessive neuronal activity can result in a 30% decrease in the volume of the extracellular space (Lux et al, 1986) and could change concentrations of extracellular ions and transmitters. A decrease in the extracellular space might be expected to increase the extracellular GABA concentration following exocytosis, and therefore increase $GABA_B$ receptor-mediated heterosynaptic depression. However, if this is accompanied by an increased tortuosity of the extracellular space, the diffusional path for GABA molecules from their release sites to the receptors could be increased, with the opposite effect on heterosynaptic depression.

In this chapter I ask:

1. Is the loss of heterosynaptic depression after status epilepticus due to a reduction in interneuron recruitment? I asked this question using two approaches. First, if

metabotropic glutamate receptor upregulation reduces interneuron recruitment, it might be possible to rescue depression by applying a group II metabotropic glutamate receptor antagonist. Second, mossy fibres make many connections with interneurons as well as CA3 pyramidal neurons. Therefore, when mossy fibres are stimulated they will recruit several different interneuron populations (Walker et al, 2002a; Henze et al, 2000; Lei and McBain, 2002) as well as providing input to CA3 pyramidal neurons. There are several interneurons that receive input from mossy fibres.

These include:

i) hilar border interneurons with ascending axons and dendrites (hilar interneuron with commissural-associational pathway-associated axon terminals, HICAP cells), with dendrites in both the hilus and molecular layer, and cell bodes in the polymorphic zone of the hilus or within *stratum granulosum* at the hilar border (Han et al, 1993; Doherty and Dingledine, 2001). They have their main axon in the hilus that gives rise to collaterals that branch in a Y-shaped manner in the molecular layer just above the granule cell layer. They mainly synapse onto dentate granule cells in the inner third of the molecular layer.

ii) mossy fibre associated interneurons that have dendrites in *stratum oriens* and *stratum radiatum,* and axons in *stratum lucidum* (Vida and Frotscher, 2000). These cells form synapses on the proximal apical dendrites of CA3 pyramidal neurons and onto other inhibitory interneurons.

iii) several interneuron types that have dendrites in *stratum lucidum* (Acsady et al, 1998) and basket cells that provide feedforward inhibition onto CA3 pyramidal neurons (Frotscher, 1985).

Since mossy fibres connect with many interneurons, I increased the length and frequency of the conditioning train in order to recruit more interneurons and hence increase GABA release. I hypothesized that if loss of depression is due to a decreased recruitment of interneurons, then increasing the train could potentially rescue depression in post-status epilepticus slices.

2. Is loss of heterosynaptic depression due to enhancement of GABA uptake? I hypothesized that if GABA uptake was increased acutely after status epilepticus, this could explain the loss of heterosynaptic depression. I therefore attempted to rescue heterosynaptic depression by interfering with the major hippocampal GABA transporter, GAT-1, with the selective blocker NO711.

6.2 Methods

I prepared acute hippocampal slices as before from animals that had had pilocarpine-induced status epilepticus 24 hours previously. I identified two separate pathways of mossy fibres as before and applied the two pathway stimulation protocol. The frequency facilitation, DCG-IV sensitivity and fEPSP ratios in naloxone only were included in the data sets presented in chapter 3. I applied the group II metabotropic glutamate receptor antagonist LY341495 (500 nM) and the GAT-1 blocker NO711 (20 μM) during the two pathway experiment to slices from animals 24 hours after

pilocarpine-induced status epilepticus and measured the fEPSP ratio with and without presence of drug. I then used another group of post-status epilepticus animals; two pilocarpine treated and two perforant path stimulated, and prepared acute slices from them. In the presence of naloxone I applied the two pathway protocol with a 50 Hz 5 pulse train as previously, and then in the same slice increased the train to 100 Hz and 10 pulses to see if this rescued depression. (These experiments were done in the presence of 4mM Ca^{++} and 4mM Mg^{++} as in all other experiments unless stated otherwise).

6.3 Results

Can depression be rescued by blocking group II metabotropic glutamate receptors? I applied the group II metabotropic glutamate receptor antagonist LY341495 (500 nM) during the two pathway experiment to slices from animals 24 hours after pilocarpine-induced SE. This concentration of LY341495 was sufficient to completely antagonise the effect of the group II mGluR agonist DCG-IV on mossy fibre transmission (n = 3, Fig. 6.1).

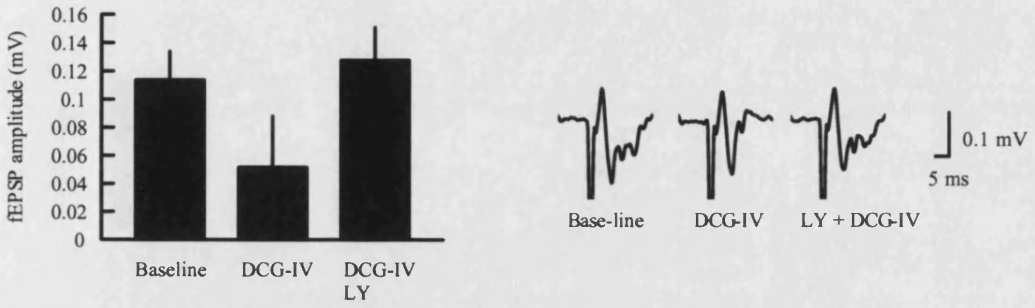


Figure 6.1: The group II metabotropic glutamate receptor antagonist LY341495 at 500nM is sufficient to block the effects of the group II metabotropic glutamate receptor agonists DCG-IV on mossy fibre transmission (n=3). Traces are averages of 5 fEPSPs in each of the three conditions. The fEPSP is abolished in the presence of DCG-IV and returns when LY is applied in the presence of DCG-IV.

LY341495 failed to rescue heterosynaptic depression (p = 0.18, paired t test, n = 5, Fig. 6.2A,B).

In the same slices, bath application of 20 μM NO711 failed to restore heterosynaptic depression (n = 4, p = 0.88, paired t test, Fig. 6.2, 6.3A). This concentration is sufficient to evoke a tonic $GABA_A$ receptor-mediated current in pyramidal neurons (Semyanov et al, 2003). These results thus lend no support to the hypothesis that

heterosynaptic depression was lost either because of upregulation of group II metabotropic glutamate receptors or because of increased GABA uptake.

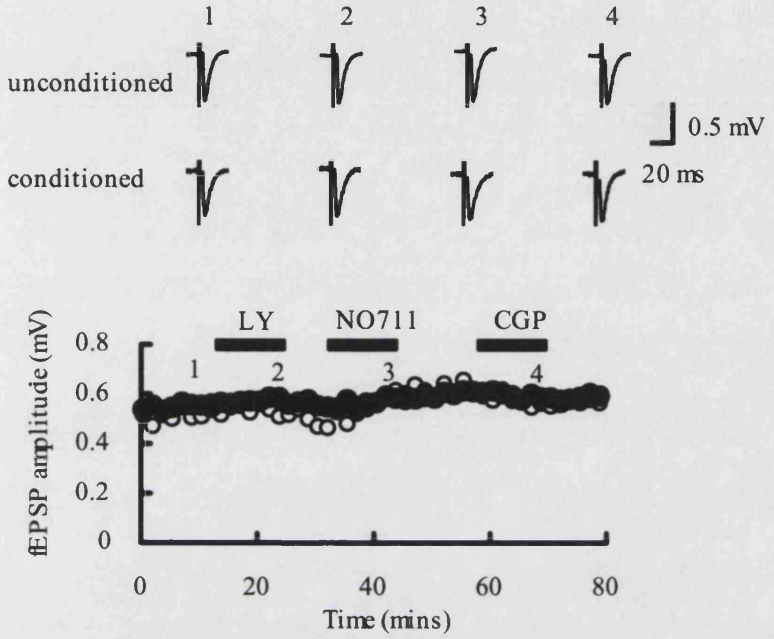


Figure 6.2: Heterosynaptic depression could not be rescued after SE by blocking group II metabotropic glutamate receptors or by blocking GABA uptake. An example of one experiment. The group II metabotropic glutamate receptor antagonist LY341495 (500 nM) failed to restore heterosynaptic depression in a slice obtained after pilocarpine-induced SE. Application of the GABA transporter GAT1 blocker NO711 (20 μM) also failed to affect the fEPSP ratio. Sample traces: fEPSPs in control conditions, and in the presence of LY, NO711 and CGP (each trace is the average of 5 trials. Conditioned fEPSPs: open circles, unconditioned fEPSPs: filled circles).

I then asked if changing the stimulation protocol to enhance the recruitment of interneurons could rescue depression. I applied the usual stimulation protocol of 5 pulses at 50 Hz. I did this in slices from two perforant path stimulated animals and in two pilocarpine treated animals. In the same slices, I increased the train to 10 pulses at 100 Hz (n = 4, pilocarpine = 2, perforant path = 2) with the aim of increasing the

number of mossy fibres stimulated and thus potentially also increasing interneuron recruitment. Heterosynaptic depression was not rescued. The fEPSP ratio was 1.0 ± 0.1 during the 50 Hz protocol and 0.97 ± 0.05 during the 100 Hz protocol. The fEPSP ratio was not significantly different between 50 and 100 Hz trains (Fig. 6.3B). This provides no evidence for the hypothesis that impaired recruitment of interneurons accounts for the loss of heterosynaptic depression post-SE.

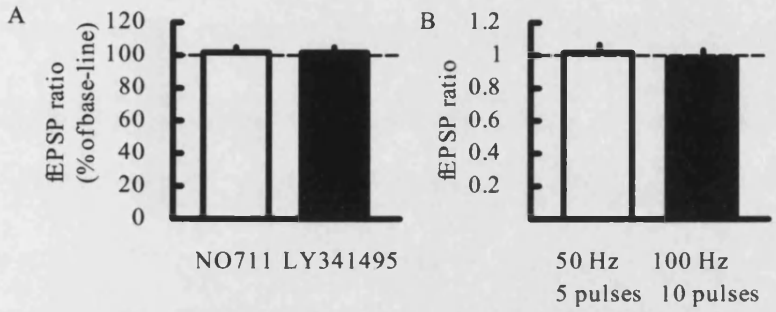


Figure 6.3: A, Summary of effects of NO711 (n = 4) and LY341495 (n = 5) on the fEPSP ratio in animals after pilocarpine induced SE. B, A 100 Hz train did not rescue heterosynaptic depression in slices after SE. The fEPSP ratio did not change significantly when the train was increased from 50 Hz to 100 Hz (p = 0.4, paired t test)

6.4 Discussion

The results presented here lend no support to the hypothesis that heterosynaptic depression is lost after status epilepticus due a loss of interneuron recruitment. The metabotropic glutamate receptor antagonist LY341495 did not rescue depression in slices after status epilepticus. The work of Doherty and Dingledine (2001) concentrated on interneurons in the border of the dentate hilus. It is possible that the upregulation of metabotropic glutamate receptors that they observed is specific to

synapses onto these interneurons and that they are not responsible for heterosynaptic depression at the mossy fibre synapse. Their observation could also be specific to the kainic acid model.

I also tried to rescue depression by using NO711 to block the GAT1 GABA transporter. This had no effect on the fEPSP ratio which suggests that an increase in GABA uptake is not responsible for the loss of heterosynaptic depression. Also, if an increase in ambient GABA does not rescue depression, it suggests that there may be insufficient functional $GABA_B$ receptors to induce depression after status epilepticus. The question of whether there are fewer $GABA_B$ receptors after status epilepticus is studied in later chapters.

Lastly, I was unable to rescue depression by increasing the frequency and length of the conditioning train. In this experiment I aimed to recruit more interneurons during the facilitation that occurs during the train applied to the mossy fibres. Depression did not return. This could be because a) there are insufficient interneurons left after status epilepticus to be recruited b) the prolonged train is insufficient to recruit them c) there is insufficient GABA in the remaining interneurons to be released. This experiment also lends no support to the hypothesis that heterosynaptic depression is lost after status epilepticus because of reduced recruitment of interneurons.
There are several limitations to this study and there are questions I did not ask regarding interneuron function following status epilepticus. I did not address the question of whether interneurons were actually lost after status epilepticus. If the

GABA that causes depression in control slices is released from interneurons this could certainly explain the loss of heterosynaptic depression. Several other studies show a loss of interneurons following status epilepticus (Houser and Esclapez, 1996; Andre et al, 2001; Obenaus et al, 1993). However, these studies observed selective loss of interneurons in *stratum oriens* of CA1 and the hilus of the dentate gyrus. The interneurons in CA3 were relatively well-preserved (Andre et al, 2001). Although I asked if there was an upregulation of metabotropic glutamate receptors that might cause reduced recruitment of interneurons, reduced interneuron recruitment could occur via other mechanisms such as loss of mossy cells (the dormant basket cell hypothesis; see General Introduction). Upregulation of other presynaptic receptors at mossy fibre-interneuron synapses such as $GABA_B$, endocannabinoid (Chen et al, 2003) or opioid receptors could also reduce interneuron recruitment. Lastly, I did not address the possibility that there is less GABA released from interneurons after status epilepticus.

Chapter 7: Are $GABA_B$ receptors downregulated after status epilepticus?

7.1 Introduction

In previous chapters, I asked if the GABA that mediates heterosynaptic depression is released from mossy fibres or interneurons. The second question that arose from my initial finding of the loss of heterosynaptic depression is: why is heterosynaptic depression lost after status epilepticus? I have started to address this question in previous chapters. I have shown that it is not due to a downregulation of GABA in mossy fibres and was also unable to demonstrate any evidence for loss of interneuronal recruitment in my models. Could the loss be due to reduced functional $GABA_B$ receptors on mossy fibre terminals?

When $GABA_B$ receptors are activated, they modulate their effector systems through activation of the inhibitory G proteins $G_{\alpha i}$ and $G_{\alpha o}$ (Couve et al, 2000; Odagaki et al, 2000). When presynaptic $GABA_B$ receptors are activated, they inhibit transmitter release. There are several lines of evidence to support the presynaptic action of the $GABA_B$ receptor agonist baclofen. First, baclofen suppresses EPSPs but does not suppress direct glutamate induced postsynaptic excitation (Howe et al, 1987). Second, if its postsynaptic actions are blocked, baclofen still suppresses EPSPs (Dutar and Nicoll, 1988). Third, baclofen can suppress release of neurotransmitter from slices into the perfusing solution (Bowery et al, 1980) and fourth the coefficient of variation of the amplitude of synaptic potentials (CV) increases

concomitantly with suppression of synaptic transmission (Harrison et al, 1990). Several of these lines of evidence are not definitive however. Quantal analysis provides a more quantitative measure of the properties of transmitter release and the drugs that affect it. A decrease in mean quantal content (m) indicates suppression of transmitter release, and a decrease in quantal amplitude (q) indicates a reduction in sensitivity of post-synaptic receptors. Inhibition of transmitter release by presynaptic receptors arises, at least in part, via a direct effect of the activated G protein on voltage-gated calcium channels (Isaacson, 1998; Kajikawa et al, 2001; Edwards et al, 1989). In addition, activation of presynaptic $GABA_B$ receptors inhibits vesicle recruitment via a reduction in cyclic AMP (Sakaba and Neher, 2003).

Mossy fibre synaptic transmission is strongly depressed by application of $GABA_B$ receptor agonists such as baclofen. However, it does have actions at both pre- and post-synaptic $GABA_B$ receptors (Hirata et al, 1992). $GABA_B$ receptors are present pre-synaptically at the mossy fibre-CA3 synapse (Vogt and Nicoll, 1999) and are also expressed on CA3 pyramidal neurons (Sodickson and Bean, 1996). Hirata (1992) used quantal analysis to study the action of baclofen on transmitter release at the mossy fibre synapse of the guinea-pig. They demonstrated both a pre- and post-synaptic action of baclofen at the guinea-pig mossy fibre synapse. Although, at the mossy fibre synapse, baclofen acts at both pre and post-synaptic $GABA_B$ receptors, most of its inhibitory effect on mossy fibre fEPSPs is likely to be via inhibition of transmitter release at presynaptic $GABA_B$ receptors. This is because the fEPSP represents the change in potential that arises in the dendrites of CA3 due to action of

glutamate on its receptors. It is possible however that a small part of its action could be due to shunting mediated by the opening of potassium channels on the postsynaptic membrane.

Here I ask: is heterosynaptic depression lost after status epilepticus because $GABA_B$ receptors are downregulated? I have used two different methods to address this question. In order to measure $GABA_B$ receptor function on mossy fibre terminals I measured the sensitivity of the mossy fibre fEPSP to the $GABA_B$ receptor agonist baclofen in control slices, and in slices 24 hours and 3 weeks after pilocarpine-induced status epilepticus. I then directly measured receptor density in *stratum lucidum* by measuring the binding of a tritiated $GABA_B$ receptor antagonist, CGP62349 using autoradiography in controls, 24 hours and 3 weeks after pilocarpine induced SE.

7.2 Methods

I prepared acute hippocampal slices as described previously (see Chapter 2: General Methods) from 3 groups of animals: controls, 24 hours, and 3 weeks after pilocarpine induced status epilepticus. The animals that had had status epilepticus 3 weeks previously were having spontaneous seizures several times/day. Twenty-four hours prior to sacrifice of the animal and slice preparation, the three week animals were intermittently observed throughout the day. If at least two clinically evident seizures were observed throughout this period that were Racine grade II or above the animal was considered to be epileptic. Ideally the animals should have had

continuous video monitoring to determine seizure frequency and severity but this type of equipment was not available in our laboratory. I recorded fEPSPs in *stratum lucidum* while stimulating a single pathway of mossy fibres in the dentate granule cell layer at low frequency (0.05 Hz). Mossy fibre fEPSPs were again identified by the magnitude of frequency facilitation and DCG-IV sensitivity. I measured the sensitivity of the fEPSP to increasing concentrations of baclofen (0.1, 1 and 10 μM).

7.2.1 Autoradiography

I performed the autoradiography experiments in the laboratory of Professor Norman Bowery at the University of Birmingham, UK. Prof. Bowery and Dr. Alessandra Princivalle instructed me how to perform these experiments but I did the majority of the practical procedures myself. The principles of autoradiography are described in Chapter 2: General Methods. Brains removed from control or post-status epilepticus rats (either 24 hours or 3 weeks following pilocarpine induced status epilepticus) were rapidly frozen in isopentane cooled in liquid nitrogen and stored at -70°C. I froze the brains at the ION and then transported them to Birmingham where I sliced them in preparation for autoradiographic labelling. Sections (12 μm thick) were cut on a cryostat (2800 Frigocut, Reichert Jung) and thaw-mounted onto Superfrost Plus slides (75 x 25 x 1.0mm; BDH), then stored at -20°C until used. On the day of binding experiments, sections were left to equilibrate to room temperature. Slides were then incubated in 50 mM Tris buffer containing 2.5 mM $CaCl_2$ (pH 7.4), for 20 minutes, before incubating in fresh buffer for 60 minutes. They were then dried in room air. Each section was then incubated for 60 minutes and subsequently dried in

[^{3}H]-CGP 62349 (at concentrations of 0.5, 0.75,1, 2, 4, and 8 nM) to determine total binding. [^{3}H]-CGP62349 at the same concentrations plus CGP54626A (10 μM) was used for incubation and then dried to determine non-specific binding. The slides were then washed in buffer for 2 x 1 minute washes and briefly rinsed in distilled water to remove buffer salts. The slides were dried in room air and then apposed to film (Amersham Hyperfilm-^{3}H). The films were developed 28 days after exposure to the slides. Quantification of receptor autoradiography was achieved by film densitometry using an image analysis system (MCID - Microcomputer Imaging Device, Imaging Research Inc., Canada), and optical density was converted to fmol/mg of bound ligand. In each Xray cassette, a standard strip was included that had previously been inoculated with different known concentrations of radioligand. Before performing image analysis the program was calibrated using this standard. The program could then convert optical density into fmol/mg of bound ligand. Total binding in *stratum lucidum* was assessed in four to eight sections per concentration of [3H]-CGP62349 for each animal. The binding parameters receptor density (B_{max}) and affinity (K_D) were determined by the use of the Langmuir equation in Prism PC software (GraphPad Software, San Diego, CA). [3H]-CGP62349 was a gift from Dr. Wolfgang Froestl, Novartis.

7.2.2 Data analysis

Data are expressed as mean ± S.E.M. The baclofen data were analysed using a least-squares fit to $I_{max}/(1 + EC_{50}/[baclofen])$, where I_{max} is the maximal inhibition and the EC_{50} is the half-maximally effective concentration of baclofen (KyPlot, Japan)

(Sodickson and Bean, 1996). Results were compared using the unpaired Student's t-test. In the autoradiography experiments, the binding parameters K_D and B_{max} were calculated for each animal using the Langmuir equation.

$Y = B_{max} * X / (K_D + X)$

(where X = concentration of radioligand applied to tissue (nM), Y = concentration of bound ligand (fmol/mg tissue).

This equation describes the binding of a ligand to a receptor that follows the law of mass action. B_{max} is the maximal binding and K_D is the concentration of ligand required to reach half-maximal binding.

7.3 Results

Baclofen reduced the amplitude of mossy fibre fEPSPs in both control and epileptic slices in a concentration dependent manner (Fig. 7.1, 7.2).

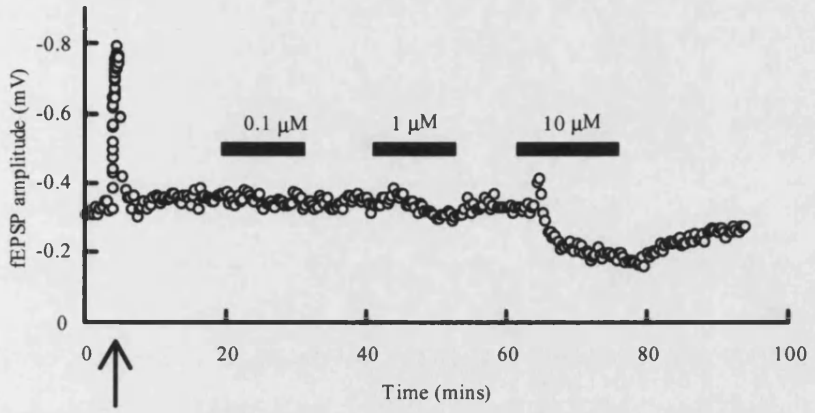


Figure 7.1: An example of an experiment in a slice from an animal three weeks after status epilepticus. At the beginning of the experiment, the frequency of stimulation was increased from 0.05 Hz to 1 Hz to confirm that it was a mossy fibre response (arrow). Then, increasing concentrations of baclofen were applied. A concentration dependent reduction in amplitude of the fEPSP was seen.

Baclofen was less potent in reducing the fEPSPs in the 24 hour post-pilocarpine SE slices, compared to control. Potency was measured by calculating the IC_{50} (controls: $IC_{50} = 0.47 \pm 0.14$ µM, n = 6; 24 hours after SE: $IC_{50} = 1.17 \pm 0.27$ µM, n = 7; $p < 0.05$ for difference; Fig. 7.2). There was no significant difference in the maximal inhibition (I_{max}) by baclofen (controls: $I_{max} = 0.95 \pm 0.05$, n = 6; SE: $I_{max} = 0.87 \pm 0.06$, n = 7; p = 0.31 for difference). I then asked whether this decrease in sensitivity to baclofen was a transient change or whether it was persistent over time. I applied baclofen to slices from animals three weeks after status epilepticus. Although the increase in IC_{50} did not reach significance when compared to controls (controls: IC_{50}

= 0.47 ± 0.14 μM; 3 weeks post-SE: IC_{50} = 0.52 ± 0.13 μM, p = 0.79), the I_{max} was significantly reduced (0.74 ± 0.05; p = 0.007, unpaired t test, Fig. 7.2) demonstrating a loss in efficacy (efficacy is defined as ability of drug, once bound to the receptor, to initiate changes which lead to effects) of the $GABA_B$ receptors.

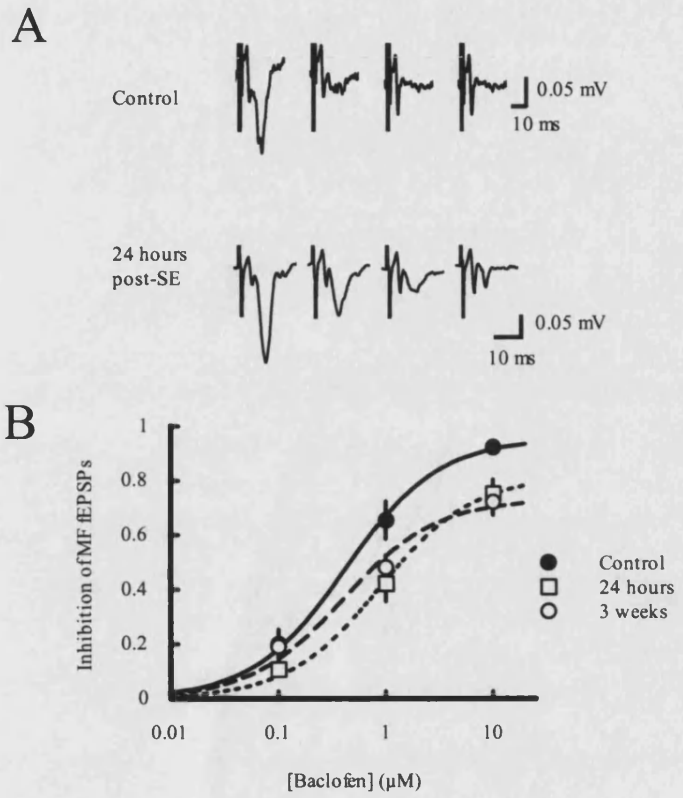


Figure 7.2: Mossy fibre fEPSPs were less sensitive to the $GABA_B$ receptor agonist baclofen after SE. A, fEPSPs (averages of 5 traces) illustrating a reduced sensitivity to baclofen 24 hours after SE. From left to right, traces are shown under base-line conditions, and in 0.1, 1 and 10 μM baclofen. These traces include the presynaptic fibre volley however all calculations were performed after the fibre volley was subtracted, as in all other experiments in this thesis. B, Baclofen has a less potent effect on mossy fibre fEPSPs 24 hours after SE ($p < 0.05$ unpaired t test on IC50 - values, 6 control slices and 7 post-SE slices) and the I_{max} was significantly rein slices 3 weeks after SE ($p = 0.007$, $n = 6$).

I then asked if the reduced potency of baclofen was due to a change in the number or functional properties of $GABA_B$ receptors. I used [^{3}H]-CGP62349 autoradiography to estimate the density of $GABA_B$ receptors in *stratum lucidum* in control and post-pilocarpine induced SE tissue (24 hours and 3 weeks after SE). The radiolabelled antagonist was applied at concentrations of 0.5, 0.75, 1, 2, 4, and 8 nM. Representative slices were stained with cresyl violet to identify the principal cell layers. *Stratum lucidum* was defined as the region between the margin of the *stratum pyramidale* of CA3 and *stratum radiatum* of CA3.

The mean K_D for post-SE animals was not significantly different from that for control animals, implying that the affinity of the $GABA_B$ receptors was unchanged (controls: 1.28 ± 0.34 nM, n = 5; SE: 1.09 ± 0.10 nM, n = 4, p = 0.67; Fig. 7.4). However, the mean B_{max} (a measure of $GABA_B$ receptor density) 24 hours after SE was significantly less than in controls in *stratum lucidum* (controls: 1322 ± 68 fmol/mg tissue, n = 5; SE: 1080 ± 56 fmol/mg tissue, n = 4; $p < 0.05$ for difference; Fig. 7.4). I also observed a non-significant trend for the B_{max} to be reduced 3 weeks following status epilepticus (1144 ± 54 fmol/mg tissue, n = 4; p = 0.09 for difference from control; Fig. 7.4).

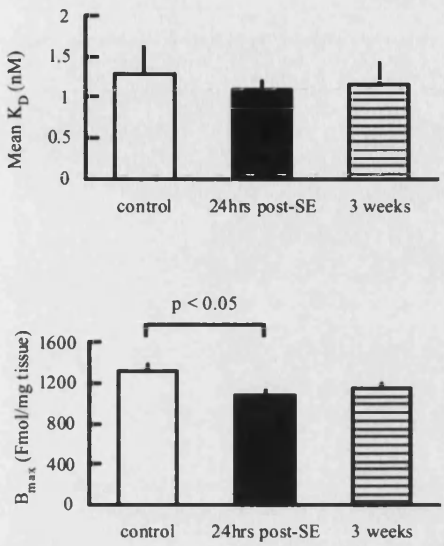


Figure 7.3: The binding parameter B_{max}, which reflects maximal binding of the radioactive ligand to $GABA_B$ receptors, was significantly lower than control 24 hours after SE (control: n = 5; SE: n = 4; p = 0.033 for difference) but not 3 weeks after SE. K_D was not significantly different either at 24 hours or at 3 weeks after SE.

Other workers have observed altered $GABA_B$ receptor function and binding elsewhere in the hippocampus. I asked if the reduction in $GABA_B$ receptor density that I observed is specific to the presynaptic $GABA_B$ receptors on mossy fibre terminals or whether it is a global change that also occurs in the dentate granule cell dendrites.

Figure 7.4: An example of an autoradiograph from an animal after pilocarpine induced SE. I measured binding in stratum lucidum, (black arrow). Stratum pyramidale of CA3 is marked with a white arrow.

In order to address this question I measured binding in the molecular layer of the dentate gyrus. The mean B_{max} and K_D 24 hours post-SE tissue were not significantly different from controls (B_{max}: controls 925 ± 74 fmol/mg tissue, n = 5; SE 812 ± 71 fmol/mg tissue, n = 4, p = 0.29; K_D: controls 0.69 ± 0.20 nM; SE 0.82 ± 0.23 nM, p = 0.83).

7.4 Discussion

The main finding of this chapter is that there are fewer presynaptic $GABA_B$ receptors on mossy fibre terminals following status epilepticus. I have demonstrated this as both a loss of function and as a reduction in receptor density. The functional change persists for at least three weeks although the change in receptor density does not reach significance at this time-point. This is certainly enough to at least partly explain the loss of $GABA_B$ receptor mediated heterosynaptic depression.

The loss of $GABA_B$ receptor function was observed as a loss in the potency of baclofen. The potency of a drug depends on its affinity, i.e. its tendency to bind to receptors, and its efficacy, i.e. its ability, once bound, to initiate changes which lead to effects. This means that the reduced potency could reflect either a loss of receptors or uncoupling from second messengers downstream of the receptor. Three weeks after status epilepticus there was still a trend towards a reduced potency but this did not reach significance. However, there was reduced efficacy measured as a reduction in I_{max}. This suggests that, at this time point, there could have been some recovery of receptor number but an increase in uncoupling of receptors from their second messengers.

A loss of binding to $GABA_B$ receptors in *stratum lucidum* could reflect reduced receptors on mossy fibre terminals. However, an 18 % loss is not sufficient to explain a total loss of heterosynaptic depression. This discrepancy could either mean that the autoradiography underestimated the loss of receptors from mossy fibre

terminals, or that other mechanisms downstream of receptor binding become dysfunctional and contribute to the loss of function. Possible downstream changes are: uncoupling between $GABA_B$ receptors and G proteins, or dysfunctional signalling between the activated G protein and voltage-gated calcium channels.

Mossy fibre terminals are not the only structures that express $GABA_B$ receptors in *stratum lucidum*. What other cells are found in *stratum lucidum*? Mossy fibre-associated interneurons are present (Vida and Frotscher, 2000) and they synapse onto the proximal apical dendrites of CA3 pyramidal neurons. If they bear $GABA_B$ receptors, they could have been detected by the autoradiographic binding. Also, an upregulation of receptors on other cell types could lead to an underestimation of the loss on mossy fibre terminals. Indeed, there are interneurons present in this area which express $GABA_B$ receptors, and increased expression of $GABA_B$ receptors in these interneurons with seizures (Kokaia and Kokaia, 2001) could mask the loss at mossy fibre terminals.

My finding that $GABA_B$ receptors are downregulated acutely after status epilepticus are consistent with other observations that $GABA_B$ receptors are altered in seizure models. Changes in $GABA_B$ receptors have been in observed in both humans with TLE and experimental models of epilepsy. In this study however, binding to $GABA_B$ receptors in the CA3 pyramidal neuronal layer is reduced in human patients with TLE (Princivalle et al, 2002) although $GABA_B$ receptors are upregulated if the data were corrected for cell loss. Binding was not measured in *stratum lucidum*. In the dentate gyrus, both presynaptic (Haas et al., 1996; Buhl et al, 1996) and postsynaptic

$GABA_B$ receptor function (Wasterlain et al., 1996) is downregulated after kainic acid induced seizures and perforant path stimulation respectively. Also, Wu and Leung, (1997) showed a decrease in efficacy of $GABA_B$ receptors on CA1 interneurons. $GABA_B$ receptors are not always downregulated in seizure models however. Gloveli et al (2003) observed an *increase* in presynaptic $GABA_B$ receptors in layer 3 of the entorhinal cortex in kindled rats. Our results are surprising in that the loss of $GABA_B$ receptors occurs so soon after SE. However, others have shown that receptors can alter rapidly after SE (Kapur and Macdonald, 1997).

By what mechanisms could $GABA_B$ receptors be lost after status epilepticus? Could the reduction be due to loss of mossy fibre terminals? This is unlikely because dentate granule cells are relatively resistant to seizures. In fact, the total surface area and active zones of mossy fibre terminals (at mossy cell connections) actually increases after seizures induced by pentylenetetrazol (Pierce and Milner, 2001). Alternatively a change in subunit composition could render the receptors unable to bind with either their endogenous ligand and/or exogenous agonist. $GABA_{B(1)}$ requires $GABA_{B(2)}$ to be trafficked to the cell membrane (White et al, 1998; Margeta-Mitrovic et al, 2000) so for instance if $GABA_{B(2)}$ failed to be expressed, functional receptors would not reach the membrane surface. Also, internalization of existing receptors following status epilepticus could explain the loss in function and binding density.

Chapter 8: The role of $GABA_B$ receptor agonism in the treatment of status epilepticus

8.1 Introduction

Status epilepticus is a life-threatening neurological emergency. Thus, the initial aim of treatment is to stop seizures as quickly as possible. This is achieved by the administration of one or more antiepileptic drugs. There are several basic mechanisms thought to be involved in the action of antiepileptic drugs (Soderpalm, 2002; Kwan et al, 2001). First, they may induce a functional block of voltage-gated sodium channels (Remy et al, 2003) (phenytoin, carbamazepine, lamotrigine, valproate, topiramate), second, they can directly or indirectly enhance inhibitory GABAergic transmission (Sills and Brodie, 2001) (benzodiazepines, barbiturates, tiagabine), third, they can inhibit excitatory glutamatergic neurotransmission (Waugh and Goa, 2003) (topiramate) and fourth they can modulate calcium ion channels (Huguenard, 1999) (ethosuximide, topiramate). Several different antiepileptic drugs have been shown to be useful in the treatment of status epilepticus. In humans, the traditional approach has been to treat initially with benzodiazepines, followed by phenytoin and barbiturates if necessary, although it has been suggested that the treatment plan should be tailored to the specific patient rather than strictly following this protocol (Lowenstein, 2003).

The antiepileptic drugs that facilitate GABAergic inhibition have several different mechanisms of action. For example, diazepam potentiates $GABA_A$ receptor responses via two pharmacologically separate mechanisms which are dependent on the concentration of diazepam used (Walters et al, 2000). The mechanism at the lower concentration depends on the presence of the γ2 subunit of the $GABA_A$ receptor whereas at higher concentrations the potentiation is independent of the γ2 subunit. The barbiturate pentobarbitone potentiates GABA-evoked responses at low micromolar concentrations and opens $GABA_A$ receptors directly at high micromolar concentrations (Serafini et al, 2000). Other antiepileptic drugs such as vigabatrin, valproate and gabapentin are believed to enhance GABAergic transmission indirectly via enzyme inhibition, causing a reduced GABA metabolism and/or promoting GABA release through unknown mechanisms (Soderpalm, 2002).

There is some evidence that the $GABA_B$ receptor agonist baclofen has anti-epileptic properties although this is not a consistent finding in all epilepsy models. It reduces epileptiform bursting in several *in vitro* models of epilepsy (Ault et al, 1986), inhibits kainic acid induced epileptiform activity *in vivo* (Sokal and Large, 2001) and prevents development of seizures in the lithium-pilocarpine model (George and Kulkarni, 1996). Furthermore, $GABA_B$ receptor *antagonists* produce seizures in normal animals (Vergnes et al, 1997). $GABA_{B(1)}$ null mice are viable and have generalized seizures (Schuler et al, 2001). By what mechanisms could baclofen be antiepileptic? $GABA_B$ receptors are present on presynaptic terminals of both GABAergic and glutamatergic cells. Therefore they can decrease inhibition and excitation depending on the site of action. Post-synaptic $GABA_B$ receptors

hyperpolarize the cell due to potassium efflux and hence reduce neuronal excitability. Presynaptic $GABA_B$ receptors, on the other hand, reduce either excitatory or inhibitory transmitter release by inhibiting voltage-gated calcium channels. In addition, $GABA_B$ receptors are altered in epileptic states as shown by this study and others. The effect of baclofen on seizures is thus difficult to predict and its mode of action as an antiepileptic drug is unclear. If baclofen has antiepileptic properties in several seizure models, does loss of $GABA_B$ receptors in my models lead to a resistance to the antiepileptic effects of baclofen? I thus asked if baclofen was antiepileptic in the perforant path and pilocarpine models of status epilepticus.

Is $GABA_B$ receptor agonism involved in the mechanisms of action of other antiepileptic drugs? Tiagabine is a relatively new antiepileptic drug which was developed from the GABA uptake inhibitor nipecotic acid (Andersen et al, 1993). It is a potent and selective inhibitor of the GABA transporter GAT-1 (Borden et al, 1994). The exact mechanisms by which GABA uptake inhibition leads to cessation of seizures are not understood. Tiagabine may enhance inhibition by increasing synaptic $GABA_A$ receptor mediated currents and/or tonic inhibition. The time course of the GABA transient in the synaptic cleft may be partly determined by GABA uptake; therefore tiagabine is able to prolong the synaptic GABA transient (Draguhn and Heinemann, 1996), although this effect is age-dependent. Increasing ambient GABA may also have some pro-epileptic effects due to desensitization of $GABA_A$ receptors (Overstreet and Westbrook, 2001) however it will also lead to enhanced

$GABA_A$ receptor mediated tonic currents (Semyanov et al, 2003) which is likely to have complex effects on network excitability. The increased ambient GABA that is produced by blocking uptake leads to increased spill-over (Rossi and Hamann, 1998) from the synaptic cleft onto extrasynaptic receptors. This will also increase $GABA_B$ receptor activation which may lead to oscillatory activity (Scanziani, 2000). Also, activation of post-synaptic $GABA_B$ receptors in the thalamus has been proposed to underlie absence seizures (von Krosigk et al, 1993). Although the antiepileptic effects of tiagabine are usually attributed to $GABA_A$ receptor activation, $GABA_B$ receptors could certainly be activated by the increased ambient GABA. Indeed, tiagabine's antinociceptive properties have been proposed to be due to $GABA_B$ receptor activation via increased GABA levels in the medial thalamus (Ipponi et al, 1999). I therefore tested the following hypothesis: the antiepileptic action of tiagabine in the perforant path stimulation model of status epilepticus is due to $GABA_B$ receptor activation. I did this by treating animals in SE with $GABA_B$ receptor antagonists before administration of tiagabine.

8.2 Methods

Status epilepticus was induced in adult rats by stimulation of the perforant path, as described in Chapter 2: General Methods. Stimulation of the perforant path was stopped after two hours. The behavioural status of the animals and the electrographic recording was monitored for ten minutes following the termination of stimulation, to ensure that the seizures were self-sustaining. Typically, electrographic discharges were observed which were characterized by the presence of spikes occurring at

least 1 Hz in frequency. The seizure severity, and frequency and amplitude of spikes was noted 10 minutes after cessation of stimulation (this is time = 0 on all figures in this chapter), and then 0.9 % saline or baclofen was administered by intraperitoneal injection. The frequency and amplitude of epileptiform spikes and seizure severity (using the Racine scale) were recorded every 10 minutes for three hours following administration of the drug. The EEG was stored on computer as described in Chapter 2: General Methods. Sedation was measured using the following scale:

0	Spontaneous movement
1	Intermittent spontaneous movement
2	No spontaneous movement
3	No response to auditory stimuli
4	Loss of corneal reflex
5	Loss of tail pinch

Table 8.1
Scale used to measure level of sedation during treatment.

8.3 Results

Twelve animals underwent perforant path stimulation. Six were used as controls and were given saline only following status epilepticus. One animal failed to go into self-sustaining status epilepticus after perforant path stimulation. Possible reasons for this could have been: incorrect positioning of the stimulating electrode during

stereotaxic placement, movement of the electrode after implantation or high seizure threshold in that particular animal. I administered baclofen to this animal ten minutes after the end of stimulation anyway, in order to see how sedative the drug was at the chosen dose (5 mg/kg) but did not include it in the final data set (Table 8.2). I chose the dose of 5 mg/kg because it had been used by others at similar dose ranges (Margineanu and Klitgaard, 2003) and this dose was shown to have physiological effects on dentate gyrus inhibition *in vivo*.

Time (mins)	**Drugs given, level of sedation observed**
0	Baclofen 5 mg/kg i/p
3	Reduced explorative behaviour
10	Reduced auditory reflex
15	No explorative behaviour, present righting reflex
30	Absent righting reaction, deeply sedated
120	Absent righting reaction, deeply sedated
180	Recovery of righting reaction, less sedation

Table 8.2
Evolution of sedation after baclofen administration in one animal that failed to go into status epilepticus after stimulation of the perforant path. Sedation was measured semiquantitatively in later experiments using the scale in Table 8.1.

Three minutes after drug administration the animal showed signs of mild sedation but was responsive to auditory stimuli. The gait in the pelvic limbs became wide-based. The auditory reflex was reduced 10 minutes after administration. At 15 minutes, the animal stopped exploring its environment and stood still in the cage although the righting reaction remained intact. (This is tested by placing the animal in lateral recumbency to see if it is able to right itself. The ability of the animal to do this depends on the visual and vestibular systems and proprioceptive function). The auditory reflex was still reduced. At 30 minutes, the righting reaction was abolished,

but muscle tone was normal. The animal had reduced postural reactions in all four limbs. At the end of the three hours of recording, the animal appeared less sedated and began to explore the cage again. It recovered uneventfully over night. I therefore used the same dose of baclofen for the next experiment.

Status epilepticus was successfully induced in the remaining five out of twelve animals. The pattern of seizures that was observed when the perforant path was stimulated was as follows: initially the animal had "wet dog shakes" which are partial seizures characterized by vigorous shaking movements. This then progressed to freezing and blank staring, rearing (Racine stage 5), falling (Racine stage 6) and occasionally "running" seizures. This is a similar pattern of seizures to that which has been observed previously when using this model in our laboratory. Baclofen was administered to one of the five animals at 5 mg/kg intraperitoneally during self-sustaining status epilepticus ten minutes after the end of stimulation. This had no significant effect on seizure activity, spike amplitude or frequency. Seizure severity remained at Racine scale 3 throughout the 3 hours of treatment.

Since 5 mg/kg had did no effect on the seizures in this animal, a dose of 10 mg/kg was administered to the remaining 4 stimulated animals. In 2/4 animals, clinically detectable seizures stopped completely two hours after treatment. By the end of the three hour recording period, one more animal had stopped seizing and the other was having stage 1 seizures only. When the data was averaged for all four animals, seizure severity was significantly reduced 2 hours after administration of baclofen

when compared to the six animals that had been administered saline vehicle only (mean seizure severity, before drug administration = 3.0 ± 0.3; 120 mins after baclofen, seizure severity = 1.0 ± 0.6, n = 4, p = 0.03 paired t test, Fig. 8.1, 8.3).

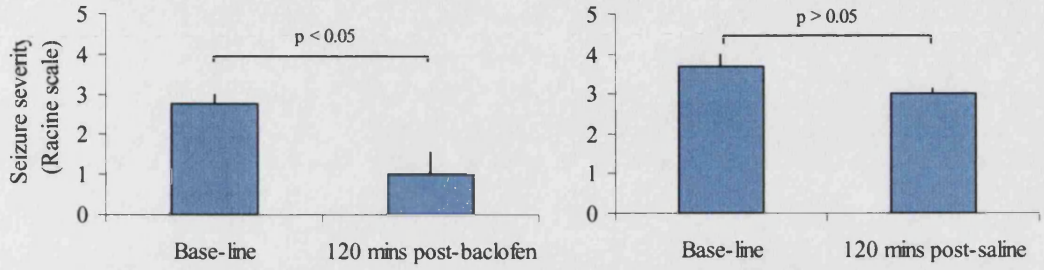


Figure 8.1: Seizure severity was significantly reduced two hours after treatment when compared to seizure severity before treatment. There was no significant reduction two hours after administration of saline.

Saline vehicle had no significant effect on seizure severity (p = 0.12 for comparison of seizure severity at 0 and 120 mins after saline, paired t test, Fig. 8.1)

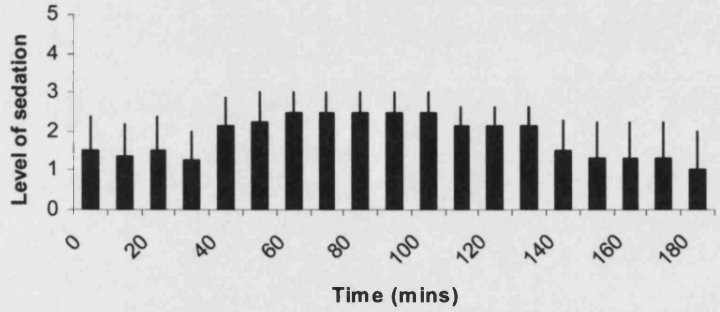


Figure 8.2: The mean level of sedation during baclofen treatment (10 mg/kg, n = 4), using the scale shown in Table 8.1.

Interestingly, the response to baclofen was extremely variable in the four animals with respect to seizure severity (Fig. 8.3). However this type of analysis is

potentially flawed because the Racine seizure scale relies on clinical observation and contains a great deal of subjectivity. Seizure types are given arbitrary numbers which correlate to the temporal evolution of seizures after kindling (Racine, 1972). One baclofen-treated animal died at the end of the recording period of unknown cause. There were no gross abnormalities seen on postmortem examination.

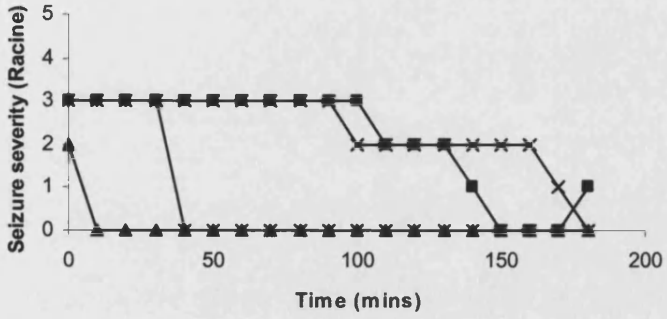


Figure 8.3: Seizure severity as measured by the Racine scale was significantly reduced two hours after administration of baclofen at 10 mg/kg but was variable in different individuals (n = 4). In 2/4 animals seizures stopped less than 50 minutes after administration of drug. (This data is summarized in Fig. 8.1 and Fig. 8.5).

The EEG was also monitored throughout the three hour period. Baclofen (10 mg/kg) reduced spike amplitude and frequency (Fig. 8.4, 8.5). The spike frequency was significantly reduced 60 minutes after drug administration (0 mins: 1.6 $\pm$ 0.17 Hz, 60 mins: 0.58 $\pm$ 0.3 Hz; $p = 0.04$ paired t test). The spike amplitude however was not significantly reduced at 60 minutes after drug administration ($p = 0.56$ paired t test), but became significant at 2 hours after drug (0 mins: 7.3 $\pm$ 1.6 mV, 120 mins: 1.8 $\pm$ 1.2 mV; $p = 0.04$). Saline vehicle did not significantly change spike amplitude when measured at 2 hours after administration (0 mins: 8.3 $\pm$ 1.5 mV, 120 mins: 7.5 $\pm$ 0.6 mV, $n = 6$, $p = 0.68$, paired t test comparing values at 0 and 120 mins).

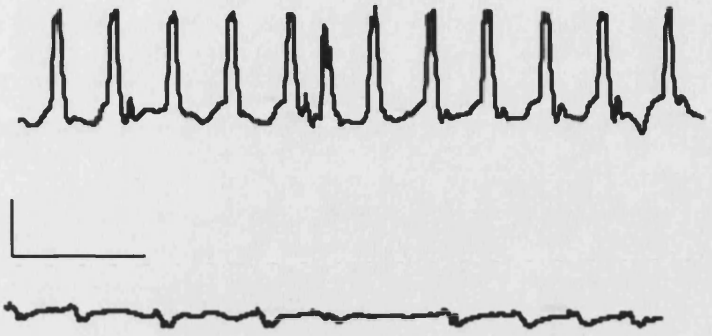


Figure 8.4: EEG traces recorded in the dentate gyrus, taken from one experiment. The top trace shows the typical spontaneous spikes that appear after stimulation is stopped, recorded just prior to drug adminstration. The bottom trace is taken two hours after administration of baclofen (10 mg/kg). Scale bar: horizontal line = 2 s, vertical line = 10 mV.

In the two animals that still had spikes at the end of the recording period, the amplitude and frequency were reduced in the middle of the experiment and then increased again, suggesting that baclofen had a transient inhibitory effect on seizure severity. This can be seen as a reduction in the activity and then an increase, in the graphs on the left shown in Fig. 8.5.

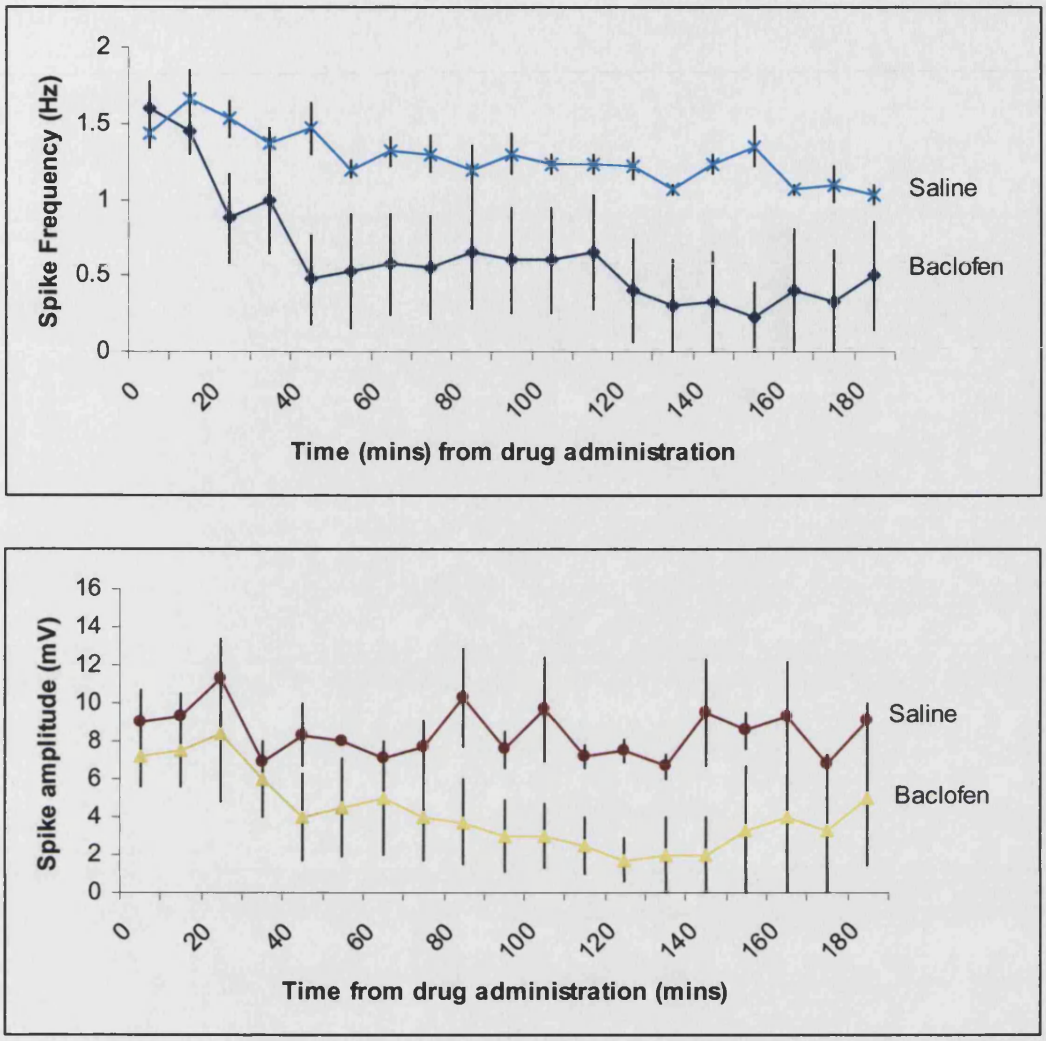


Figure 8.5: Baclofen significantly reduced spike amplitude and frequency when compared to saline vehicle. The top panels show spike frequency plotted against time in baclofen (n = 4) (10 mg/kg) and saline (n = 6). The bottom panel shows spike amplitude against time in baclofen and saline (in the same animals as top panel).

I then asked if baclofen also had antiepileptic effects in the pilocarpine model of status epilepticus. I induced status epilepticus with pilocarpine in 4 animals as described previously. These animals had had a recording electrode implanted in the dentate gyrus (using the same stereotaxic methods used for the perforant path model). After 90 minutes of status epilepticus I administered baclofen (10 mg/kg

i/p). Three out of 4 animals became deeply unconscious and showed increased respiratory effort and so I euthanased them on humane grounds before the end of the experiments. In the 4th animal, behavioural seizures stopped completely, 10 minutes after the administration of baclofen. This animal recovered well. No further experiments were done using pilocarpine and baclofen because of the high level of mortality.

I next asked if the antiepileptic efficacy of tiagabine in status epilepticus could be explained by its effect on $GABA_B$ receptors. Tiagabine has been previously shown to be antiepileptic in our perforant path model of status epilepticus, in all animals tested (X Wang, Institute of Neurology, unpublished data).

I induced status epilepticus in 3 rats by stimulation of the perforant path. Ten minutes after the cessation of stimulation, I administered the $GABA_B$ receptor antagonist SCH50911 (10 mg/kg n = 2, 40 mg/kg n = 1) and then 10 minutes later treated them with tiagabine (40 mg/kg). I did not observe any change in behaviour or seizure activity after administration of SCH50911. Within approximately 30 minutes of administration of tiagabine, seizure activity was reduced in all animals (Fig. 8.6). In both animals, the spike amplitude reduced after 20 minutes and had ceased completely after 40 minutes. This is similar to previous data from our laboratory using tiagabine as an antiepileptic drug without pre-treatment with SCH50911.
It can therefore be concluded that SCH50911, either at 10 mg/kg or 40 mg/kg, does not prevent the antiepileptic effect of tiagabine.

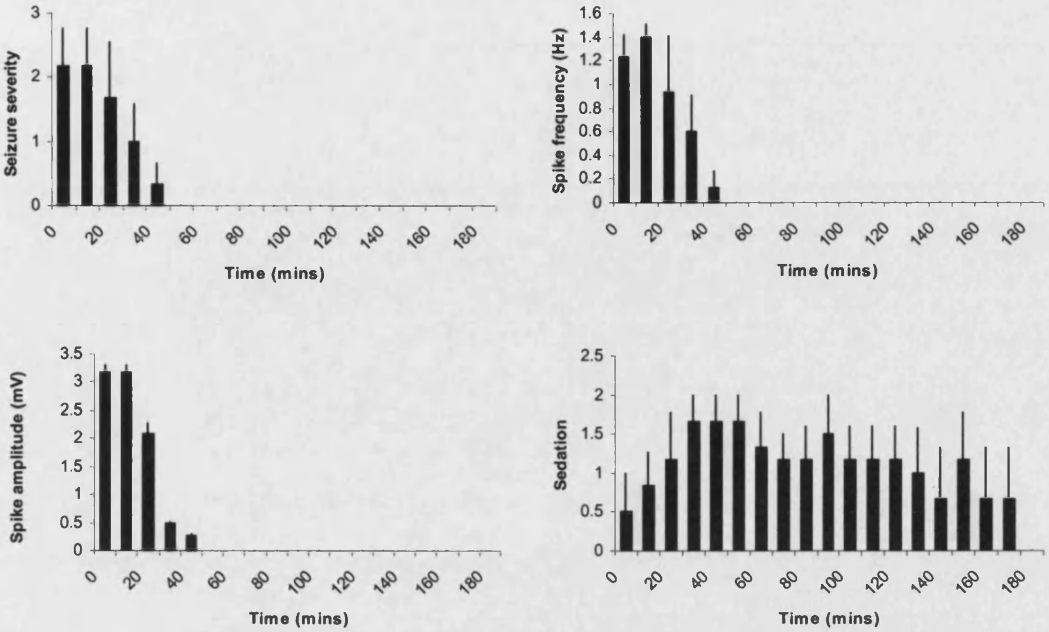


Figure 8.6: Seizure severity, spike frequency and amplitude and sedation were measured during treatment of animals in status epilepticus with tiagabine that had been pre-treated with SCH 50911. Pretreating with the $GABA_B$ receptor antagonist SCH50911 (10 and 40 mg/kg) did not affect the efficacy of the antiepileptic drug tiagabine (40 mg/kg). Tiagabine had a clear effect on seizure activity which started approximately 20 minutes after administration.

8.4 Discussion

Baclofen had a weak anti-epileptic effect in the perforant path model of status epilepticus although it took a minimum of one hour to reduce significantly seizure activity. Its effect was variable in different individuals and in two animals there were was only a transient reduction in seizure severity and spike amplitude and frequency. When the data were averaged, there was no significant effect on seizure severity for at least two hours. The antiepileptic effect is consistent with the work of others in the kainic acid (Ault et al, 1986) and lithium-pilocarpine models (George and Kulkarni, 1996).

One perforant path-stimulated animal died of unknown cause three hours after administration of baclofen. Occasionally perforant path stimulated animals die unexpectedly; usually if the seizures are very severe. Likely causes of death in status epilepticus are: abnormalities within the brain such as cerebral oedema or ischaemia, or conditions in other body systems such as aspiration of gastric contents, cardiorespiratory arrest, pulmonary oedema or metabolic acidosis.

In the pilocarpine model, there was a 75 % mortality rate after the administration of baclofen and so no further experiments using this protocol were done. Pilocarpine-induced seizures tend to be more severe than those induced by perforant path stimulation. The animals are often weak for several hours afterwards. Systemic effects such as dehydration, acid-base disturbances and pulmonary oedema may have been possible causes of death in these animals. Could baclofen itself have contributed to mortality in these experiments? Baclofen is used in humans with conditions such as multiple sclerosis, spinal cord disease, cerebrovascular accidents or neoplastic or degenerative brain disease, to control spasticity. It is reported to be toxic in patients with renal failure because it is primarily excreted through the kidneys (Chen et al, 1997). Baclofen overdose has been reported (Chapple et al, 2001). This is characterised by severe respiratory depression, coma and brainstem dysfunction. In humans these symptoms may be further aggravated by other agents that act on the CNS. It is therefore possible that baclofen contributed to the high mortality in these animals. Since baclofen takes 1-2 hours to have an effect on status epilepticus, this prolonged seizure time could also have contributed to the animals'

death. A direct interaction between pilocarpine and baclofen also cannot be ruled out.

Why was there only a transient antiepileptic effect seen in two animals? This could have been for several reasons. First, the pharmacokinetics of baclofen could be such that the drug is redistributed or metabolised more rapidly in some individuals. Also, $GABA_B$ receptors could become resistant to baclofen later in status, similar to $GABA_A$ receptor resistance to benzodiazepines late in status epilepticus (Kapur and MacDonald, 1997) and this may be variable within individuals. Changes in other receptor function may occur later which induces hyperexcitability in the brain that is resistant to baclofen.

$GABA_B$ receptor activation is unlikely to play a significant role in the antiepileptic effects of tiagabine in this model. First, the efficacy compared to the degree of sedation for tiagabine was very different from that observed for baclofen. Second, pre-treatment with the $GABA_B$ receptor antagonist SCH50911 did not affect the antiepileptic properties of tiagabine. This suggests that its antiepileptic effect in this model is mainly via $GABA_A$ receptors. The highest dose of SCH50911 that I used was 40 mg/kg. It is possible that this was not high enough to block receptors sufficiently to have a significant effect on the action of tiagabine. However, 10 mg/kg given subcutaneously is sufficient to reduce the physiological effects of baclofen in rats (Phillis et al, 2001) and doses as low as 2.9 mg/kg are sufficient to block the anti-tussive effects of baclofen in guinea-pigs (Bolser et al, 1995). Ideally,

the brain concentration needs to be measured directly to ensure there is a sufficient concentration to antagonize the tiagabine, by using a method such as microdialysis.

From this study, I propose that $GABA_B$ receptors may be a useful target for antiepileptic drugs. Baclofen was antiepileptic in the perforant path model, even though $GABA_B$ receptors are downregulated. It should be noted however, that the drug was tested in animals during status epilepticus and the downregulation of $GABA_B$ receptors that I observed occurred 24 hours after the termination of status epilepticus. The $GABA_B$ receptors may still be functioning normally at the time that baclofen was given. Baclofen took 2 hours to have a maximal effect which is too long to be useful clinically in the treatment of status epilepticus. I did not try higher doses in this study because it was already markedly sedative at 10 mg/kg. Since the number of animals tested with baclofen was small, more animals need to be tested to make firm conclusions from these data.

Other studies have shown that baclofen can be both pro- and antiepileptic depending on the model used. The antiepileptic effects of baclofen may be unpredictable in different models for several reasons. $GABA_B$ receptors are present pre- and postsynaptically, and presynaptic receptors inhibit transmitter release on both excitatory and inhibitory cells. This means baclofen can be pro- or anti-epileptic depending on its site of action. The drug is given intraperitoneally and it is not known how much of this reaches the brain, and whether it reaches higher concentrations in different brain regions. It is, however, known to cross the blood-

brain barrier (Deguchi et al, 1995). It may be more effective if it is given intravenously, intrathecally or directly onto the brain. As discussed in previous chapters, the expression and function of $GABA_B$ receptors is altered in the epileptic brain. This too will change the effect that baclofen has on network excitability. It is therefore difficult to predict the anti-epileptic effect of baclofen because of the complex changes in $GABA_B$ receptor expression and multiple sites of action.

Chapter 9: General Discussion and Further Work

9.1 Summary of Findings

In this thesis I have achieved the following:

- I have established a protocol for inducing status epilepticus with pilocarpine that achieves a high success rate with a low mortality.
- I have characterised heterosynaptic depression in the adult rat mossy fibre – CA3 synapse
- I have demonstrated that heterosynaptic depression is altered after status epilepticus
- I have addressed the question of why heterosynaptic depression is altered after status epilepticus and have attempted to determine the source of GABA that mediates the depression
- I have explored the role of $GABA_B$ agonism in the treatment of status epilepticus by a) testing the antiepileptic effects of baclofen in my models of status epilepticus and b) asking if the action of the antiepileptic drug tiagabine is partially mediated by $GABA_B$ receptors

9.2 Limitations of this thesis

How are my findings relevant to naturally occurring epilepsy in humans? It is not known whether heterosynaptic depression at the mossy fibre synapse occurs *in vivo*, and if so, whether it is absent in epilepsy. If such a depression occurs between adjacent mossy fibre pathways in the living animal, then loss of heterosynaptic depression could lead to hyperexcitability of the hippocampus in the epileptic human or animal. However, regardless of whether such a depression occurs between mossy fibres *in vivo,* my findings clearly show a loss of $GABA_B$ receptors which is extremely likely to have an effect on network excitability. The $GABA_B$ receptor has not yet been studied specifically at the mossy fibre synapse in humans, although changes in $GABA_B$ receptors have been observed at certain regions in the epileptic hippocampus, which are discussed later.

$GABA_B$ receptors were only studied at one synapse in the hippocampus, but they occur both pre- and post-synaptically throughout the brain. Changes at other sites need to be taken into account in order to determine the effect on the network. Since there is plasticity of numerous other receptors, neurotransmitter systems, and axons in epilepsy, my data need to be interpreted in this context.

I only studied $GABA_B$ receptors up to 3 weeks after an episode of status epilepticus. Heterosynaptic depression was only studied 24 hours after status epilepticus. It was not possible to identify two separate mossy fibre pathways after this time point,

presumably because of axonal reorganisation in the dentate gyrus (mossy fibre sprouting). Three weeks after status epilepticus I observed that $GABA_B$ binding partially recovered. However the baclofen data revealed that a significant reduction in efficacy of $GABA_B$ receptors remained. Taken together this suggests that an uncoupling of receptors from their second messengers could have occurred at this late time point. I did not investigate this possibility in this study.

I was unable to confirm definitively that the GABA that mediates heterosynaptic depression is released from interneurons rather than mossy fibres. Other approaches that might have yielded a definitive answer are discussed at the end of this chapter.

One important point that has arisen from this thesis is the difficulties in investigating a disease in which a universal animal model does not exist. Epilepsy is not a homogeneous condition and it is therefore difficult to produce an ideal model. In the existing models, the animal suffers some sort of insult: e.g. chemical, electrical, genetic mutation, which leads to a propensity to have seizures. The models can then be justified to a greater or lesser degree by comparing such parameters as seizure type and brain pathology to the human diseases. If these parameters are similar, they are considered to be a good model. In these models, the outcome of the insult appears similar to the human disease but the insult is very different in the model compared to humans, e.g. pilocarpine or kainic acid administration. In fact, in human epilepsies such as temporal lobe epilepsy, the aetiology is not fully understood anyway. To what extent then, are we justified in calling them appropriate

models? One way of testing them out, is to see how seizure models behave when they are treated with antiepileptic drugs. Several of these models are useful for the preclinical tests of antiepileptic drugs. However, interestingly and not surprisingly, different models respond differently to antiepileptic drugs. A comparison of chronic epilepsy models, such as the post-status epilepticus spontaneous seizure models, with the acute models such as maximal electroshock, demonstrates that drug testing in chronic models of epilepsy yield data which are more predictive of clinical efficacy and adverse effects. In fact, if chronic models such as fully kindled rats and spontaneous recurrent seizures in post-status models are compared to each other, the pharmacological properties of the seizures, i.e. the way they respond to antiepileptic drugs, is remarkably similar. The acute models on the other hand have a number of problems. The maximal electroshock test preselects drugs with certain mechanisms of action but misses others (Meldrum, 1997). This is because electroconvulsive seizures are particularly sensitive to drugs that block sodium channels. Differences in models are also problematic for the study of the effect of drugs on the epileptogenic process. This arises presumably because since each model is produced by a different insult on the brain, the epileptogenic process could occur via different mechanisms. The *in vitro* models of seizures are also used widely. Can they be considered to be useful models for human diseases? They usually consist of slices of hippocampus or hippocampus plus entorhinal cortex and they are perfused with artificial CSF either containing a convulsant such as pilocarpine or 4-aminopyridine, or solution containing zero Mg^{++} in order to produce epileptiform activity. The epileptiform activity can then be monitored by recording field potentials in different

parts of the slice simultaneously or by measuring the intrinsic optical signal (Avoli et al, 2002). The latter are produced by changes in light scattering and absorption that reflect swelling of neurons and glia leading to alterations in the extracellular space volume. The spread of epileptic discharge can be monitored across the slice, presumably because as cells depolarise they become swollen. Certainly *in vitro* models of seizures are useful to ask questions in epilepsy research but epileptiform activity in a slice is not necessarily analogous to an epileptic seizure in the whole animal. Epilepsy is a network phenomenon. We study it in slice preparations, computer simulations and in single channels expressed in cell systems such as *Xenopus* oocytes. The study of a thin slice of brain, receptors expressed in single cells or simple mathematical models of neural networks is not sufficient to understand fully how and why a seizure arises and propagates through a whole brain. It is important to be aware of this and to work towards combining all of these approaches with work done *in vivo*.

How do we get around these problems? I tried to avoid it by using two very different models of status epilepticus. Finding the same change in the two models makes the result more likely to be relevant to naturally occurring epilepsy. How else could we ensure that we are using appropriate models? More work, particularly electrophysiological studies, needs to be done using tissue from human epileptic patients. It is now possible to make recordings from fresh human tissue. For example, Cohen et al (2002) recorded synchronous rhythmic activity in tissue resected from temporal lobe epilepsy patients that was very similar to interictal

activity recorded *in vivo*. It is also now possible to express channels originating from fresh human tissue in simple systems such as Xenopus oocytes. This has been achieved both by injecting human mRNA (Palma et al., 2002) and neuronal membrane (Palma et al, 2002; Miledi et al, 2002) into oocytes. This has resulted in comparisons of channels from epileptic tissue with that from control human tissue. However, in human studies control tissue is difficult to obtain. Palma et al (2002) used tissue from non-epileptic patients who had temporal lobe glioma. Clearly it is not ideal to use tissue from patients with other neurological disease because there could be expression of abnormal channels in the diseased tissue. Finally, the ideal way in which to study synaptic function in epilepsy, is to make cellular recordings *in vivo*. Patch clamping now has been achieved *in vivo* (Khazipov and Holmes, 2003; Leinekugel et al, 2002).

9.3 Possible mechanisms to explain my findings

Why is there an apparent species difference in heterosynaptic depression?

In chapter 3, I demonstrated that heterosynaptic depression is mediated solely by $GABA_B$ receptors in adult rats. This differs from the situation in the guinea-pig in which there is a group I/II metabotropic glutamate receptor mediated component (Vogt and Nicoll, 1999; Min et al, 1998). Why should this be the case? It could either be due to different receptor subtypes or amount of receptors on mossy fibre terminals, or less glutamate spillover in rats compared to guinea-pigs. Is there evidence for any of these possibilities? There appear to be subtle pharmacological differences in metabotropic glutamate receptors at guinea pig and rat mossy fibre

synapses (Henze et al, 2000). Lanthorn et al (1984) observed that the specific group III metabotropic glutamate receptor agonist L-AP4 inhibits transmission at the guinea-pig but not rat mossy fibre synapse although rat mossy fibre synapses are sensitive to the group II/III metabotropic glutamate receptor agonist DCG-IV (Kamiya et al, 1996). This suggests that the rat expresses group II receptors but does not express group III receptors at the mossy fibre synapse. It could also be a difference in receptor expression in juvenile and adult animals, since Vogt and Nicoll (1999) and Min et al (1998) used 5-15 day old guinea pigs in their experiments. Could the findings of Vogt and Nicoll (1999) and Min et al (1998) be attributed to group I receptors and if so, could they be absent on rat mossy fibres? This is very unlikely because group I metabotropic glutamate receptors are usually considered to be excitatory and have not reported to induce presynaptic inhibition at the mossy fibre synapse. Group I metabotropic glutamate receptors are expressed post-synaptically at the mossy fibre – CA3 synapse (Yeckel et al, 1999). Could it then be due to a species difference in spillover? This is certainly possible. Extracellular glutamate concentration is determined by three processes. Differences in: amount of vesicular release into the synaptic cleft, diffusion from the extracellular pool, the size and tortuosity of the extracellular space and glutamate uptake mechanisms could all contribute to species variation in the degree of spillover.

How can complex changes in receptor expression be explained?

Another interesting question is: if *in vivo*, $GABA_B$ receptors are both up and downregulated at different synapses, as well as changes in other types of receptors, how does this complex set of changes arise? For example, does altered $GABA_B$ receptor expression in the dentate gyrus affect the transcription and translation of $GABA_B$ receptor genes in CA1, and if so, by what mechanisms is it controlled? If excitability is increased at one synapse, does a gating phenomenon come into play at another synapse? How could such communication occur? Communication through axonal transport and neurotransmitter or peptide signalling molecules would be possible mechanisms. These kinds of phenomena could arise as physiological mechanisms that stop seizure activity (and then fail when seizures develop into status epilepticus). Certainly there is evidence of markedly altered gene expression during epileptogenesis in epilepsy models. Hendriksen et al (2001) used serial analysis of gene expression (SAGE) to study changes in gene expression after perforant path stimulation. They found 92 genes which were differentially expressed compared to controls.

9.4 Implications of my work

$GABA_B$ receptors are altered in temporal lobe epilepsy

My work demonstrates a loss in functional $GABA_B$ receptors following status epilepticus induced by two different stimuli. How does this finding add to what is already known about changes in $GABA_B$ receptors in epilepsy? $GABA_B$ receptors are already known to be involved in the pathogenesis of absence epilepsy, as

discussed previously. There is now burgeoning evidence that alterations in $GABA_B$ receptors play a role in temporal lobe epilepsy. Table 9.1 below summarises the findings of the studies that have reported changes in $GABA_B$ receptors in temporal lobe epilepsy and models of the disease. $GABA_B$ receptors are either up- or down-regulated depending on the model and site studied. However a downregulation is more commonly observed than an upregulation. Two studies in humans with TLE report a downregulation of receptors. Is it possible then that the models in which an upregulation is observed are not particularly good models of the human disease? In contrast, Furtinger et al (2003b) report an increase in $GABA_B$ receptor mRNA in the dentate granule cells of patients with TLE. They also observed a marked increase of mRNA in the dentate hilus in TLE patients with non-sclerotic pathology. The same group reported a downregulation of $GABA_B$ receptor mRNA in granule cells and CA3 5-12 hours after kainate induced seizures in rats which recovered to control levels in CA3 and was upregulated in dentate granule cells at later time points (Furtinger et al, 2003a). It appears then, to complicate matters further, that changes in receptor expression vary throughout the course of the disease.

Table 9.1 : Summary of changes reported in $GABA_B$ receptors in TLE and experimental models

Author, date	Site, cell type, pre- or post-synaptic	Experimental model/ human disease	Up- or down-regulation of receptors	Proposed effect on excitability
Haas et al, 1996	DG, interneurons, presynaptic	Kainic acid	Down-regulation	Increased inhibition
Buhl et al, 1996	DG, interneurons, Presynaptic	Perforant path	Down-regulation	Increased inhibition
Wasterlain et al, 1996	DG, dentate granule cells, postsynaptic	Perforant path	Down-regulation	Decreased inhibition onto DG cells
Wu and Leung, 1997	CA1, interneurons, presynaptic	Partial hippocampal kindling	Down-regulation	Increased inhibition
Kokaia and Kokaia, 2001	DG, CA1-CA3	Rapid kindling	Mixed. Early up-regulation	? decreased inhibition
Princivalle et al, 2002	DG, hilus, CA3	Humans TLE	Down-regulation	Decreased inhibition
Munoz et al, 2002	DG	Humans TLE	Down-regulation	Decreased inhibition
Gloveli et al, 2003	ER cortex layer III, interneurons, presynaptic	Amygdala Kindling	Up-regulation	Enhanced activation of EC-CA1
Chandler et al, 2003	Mossy fibre terminals, presynaptic	Pilocarpine and perforant path	Down-regulation	Increased excitation into CA3
Straessle et al, 2003	CA1, CA3, DG	Mouse intracerebral kainic acid	Early down-regulation, recovery later	Decreased inhibition
Furtinger et al, 2003	CA1, CA3, DG	Humans TLE	Mixed. Up-regulation mRNA, down-regulation receptor binding	Increased inhibition
Furtinger et al, 2003	CA3, DG	Kainic acid	Early down-regulation, recovery later	Decreased inhibition

What is the global effect of these changes in $GABA_B$ receptors on excitability?

$GABA_B$ receptors are present both pre and post synaptically. Presynaptic receptors inhibit transmitter release at both inhibitory and excitatory neurons (Lei and McBain, 2003) and post-synaptic receptors cause hyperpolarisation by opening potassium channels (Gage, 1992). This means that they can decrease inhibition or increase excitation depending on their location. I have summarised the proposed effects on excitation in Table 9.1. The role of $GABA_B$ receptors in limbic network excitability has also been studied in *in vitro* models of epileptiform activity. Avoli et al (2004) recently reported that baclofen inhibits interictal activity in 4-aminopyridine treated entorhinal cortex-hippocampal slices. Baclofen inhibited ictal activity originating in entorhinal cortex and initiated ictal activity in CA3. The ictal activity in the absence of baclofen was NMDA receptor dependent and was NMDA independent in the presence of baclofen. Hence, in this slice model of epileptiform activity, modulating $GABA_B$ receptors altered the site of initiation of ictal events and changed the receptors that were involved. The net effect of changes in $GABA_B$ receptors on the hippocampal network is still unclear. It is extremely difficult to predict the net effect of $GABA_B$ receptor plasticity on seizure propagation through the hippocampus. However, more studies show reduced $GABA_B$ receptors which tend to be associated with increased excitability.

From this complex situation, several questions arise. First, how could we determine the role of $GABA_B$ receptor changes at each site in the hippocampus? There are several ways that we could address this question. If we could selectively alter the expression of receptors at specific synapses, we could then observe the net effect on excitability. This might be possible using transgenic technology such as the cre/lox system. *Cre* is a gene that encodes for a "site-specific recombinase". The *cre* protein can recombine DNA when it locates specific sites in a DNA molecule. These specific sites are known as *loxP*. Transgenic mice containing a gene surrounded by *loxP* sites are mated with transgenic mice that have the *cre* gene expressed only in one cell type. The resulting mice will have both the *cre* gene and the *loxP*-flanked gene. In tissues with no *cre* gene the target gene with be present and function normally. In a cell where *cre* is expressed, the target gene of interest will be deleted. Therefore, if the *cre* gene is bound to a promoter that only allows *cre* production in neuronal cells, the target gene will only be deleted in those cells.

This technology can already be used to knock out genes from specific parts of the brain such as the hippocampus. If *cre* could be expressed in specific neuron types in the hippocampus, it would be possible to prevent the expression of $GABA_B$ receptors at specific synapses and determine the effect on excitability. However, this relies on identifying a gene that is only expressed in the neuron type in question (i.e. dentate granule cells). A line of cre-transgenics has already been produced in which the cre-mediated gene deletion is restricted to granule cells of the cerebellum and dentate gyrus of the hippocampus (Guo et al, 2000).

An alternative approach would be development of a mathematical model of the hippocampal circuitry. It would then be possible to incorporate changes in $GABA_B$ receptor function at specific synapses. If we were able to determine which receptor alterations were the most important, theoretically we could then target antiepileptic drugs at specific synaptic sites; either by using local application of drug, or by using pharmacological differences in receptors at different sites to target a drug to a specific synapse.

The relevance of my findings to temporal lobe epilepsy in humans

Are my findings relevant to human disease? What is the evidence that $GABA_B$ receptor plasticity has any role in human epilepsies? Altered expression of $GABA_B$ receptors has been observed in tissue from human patients (Princivalle et al, 2002; Munoz et al, 2002; Billinton et al, 2001). Also, a polymorphism in the $GABA_{B(1)}$ gene has recently been associated with an increased susceptibility to temporal lobe epilepsy and it may be associated with a more severe phenotype of the disease (Gambardella, 2003). As discussed in detail in the introduction, $GABA_B$ receptor activation also plays a very important role in setting the oscillatory rhythm 3 Hz spike-wave absence seizures. Taken together, there is certainly burgeoning evidence that $GABA_B$ receptors play an important role in human epilepsy. Could $GABA_B$ receptors be a useful target for antiepileptic drugs? $GABA_B$ receptor agonists have both pro- and anti-convulsant properties in experimental models, as discussed in the introduction. Also, Kofler et al (1994) reported three human patients that had seizures following intrathecal baclofen administration. However, these patients had

previously suffered brain trauma which could have lowered their seizure threshold. From my work and those of others then, $GABA_B$ agonism has a complex inconsistent effect on the excitability of the network. Some factors that might lead to this inconsistency are the nature of the network itself (brain slice compared to whole brain) and the nature of any seizure activity (i.e. the model used). The effect may vary in different individual humans or animals, due to differences in genetic background.

Heterosynaptic depression and memory

Heterosynaptic depression is an important mechanism for gating input into the hippocampus proper and is therefore in a position to prevent seizure propagation. Vogt and Nicoll (1999) also suggest that it is important because it makes the input into CA3 more sparse. CA3 is a highly interconnected autoassociative network. It is proposed that such networks store patterns of inputs and retrieve them – if presented with a part of a learned pattern, the full pattern is returned. Sparse coding will increase the storage capacity of the autoassociative network. The loss of depression also has implications for memory function. Heterosynaptic depression makes coding into CA3 more sparse and if it is not present, then associative memory in the CA3 network could be affected. Cognitive deficits, including amnesia ("transient epileptic amnesia"), are associated with temporal lobe epilepsy. It is not clear whether these episodes are seizures or post-ictal abnormalities. The characteristics of this memory impairment are variable. Zeman et al (1998) reported that all patients in their study had retrograde amnesia, including loss of recall of salient episodes from their

personal lives. Some also had topographical amnesia, with failure to recognise familiar landmarks and to recall familiar routes. The loss of heterosynaptic depression could be one reason why memory recall can be abnormal.

The evolution of the theories about epilepsy: current ideas

Epilepsy is often referred to as being an imbalance between excitation and inhibition (Dalby and Mody, 2001). This theory has emerged because if inhibition is decreased by blocking $GABA_A$ receptors for example, seizures occur (Fisher et al, 1989), drugs that potentiate $GABA_A$ receptors are antiepileptic (Soderpalm, 2002) and increased excitation can be observed in the epileptic brain. However, this hypothesis does not always hold true. It is now known that rhythmic inhibitory interneuronal activity is involved in oscillatory phenomena in the hippocampus (see Chapter 1: General Introduction). Does this mean that increased inhibition can also be involved in epilepsy? Another common idea is that epilepsy arises when synchronization of neuronal firing *increases*. However recent work proposes that synchronization decreases just before a seizure occurs (Mormann et al, 2003). In very recent years, several mutations in ion channels have been associated with idiopathic generalised epilepsies in humans. These include mutations in the genes that encode for subunits of the $GABA_A$ receptor (Wallace et al, 2001a; Baulac et al, 2001), sodium channels (Wallace et al, 2001b; Wallace et al, 1998), potassium channels (Singh et al, 2003) and nicotinic acetylcholine receptors (Steinlein et al, 1995). The functional abnormality that ensues from the expression of these abnormal channels is a fault in excitability, thus leading us back to the imbalance between excitation and inhibition.

These types of observations seem to shed light onto how the normal brain may become epileptic. However, it is not quite as simple as that. People who express these abnormal channels do not necessarily have seizures as part of their phenotype. For example, episodic ataxia type I, which is caused by a mutation encoding the gene for the potassium channel Kv1.1, is characterised by gait abnormalities either with or without seizures. Why do people expressing this abnormal channel not always have seizures? There are several possible explanations for this variable phenotype. First, perhaps other inhibitory phenomena are able to come into play to prevent seizures in some individuals. Second, does the genetic background of the individual alter seizure threshold? Taken together, what does this tell us about epilepsy? The excitability of the brain can be altered by several different insults and phenomena. It is associated with changes in circuitry e.g. mossy fibre sprouting, changes in channel function e.g. sodium channels with altered kinetics and changes in receptor expression e.g. downregulation of $GABA_B$ receptors on mossy fibres. These types of alterations can "tip the balance" and induce seizures. However, the brain has mechanisms to stop seizures, which is why most seizures only last a maximum of a couple of minutes, and status epilepticus rarely develops. Even the presence of a mutated ion channel can apparently be compensated by other unknown phenomenona.

9.4 Further work

I would like to determine whether the GABA that mediates heterosynaptic depression is definitely released from interneurons and not mossy fibres. I could do

this using a whole cell recording approach. A slice could be perfused with solution containing AMPA/kainate receptor antagonists. Since interneurons express AMPA and kainate receptors which depolarise the cell if activated (Toth and McBain, 1998; Freund and Buzsaki, 1996), this will prevent interneuron recruitment. NMDA receptor mediated EPSCs can then be recorded in CA3, while the two pathway protocol is performed. If depression still occurs, then the GABA must be being released from the mossy fibres since interneurons will not be recruited.

In order to be sure that tiagabine does not mediate its effect by $GABA_B$ receptors I need to perform more experiments that ensure that the $GABA_B$ receptor antagonist reaches the $GABA_B$ receptors in sufficient concentration to block them. In the experiments in Chapter 8, the concentration of the drug that reaches the brain is not known. I could confirm that $GABA_B$ receptors are not activated by tiagabine in the following ways: I could induce seizures in an anaesthetized animal while monitoring the EEG. I could then apply the antagonist directly onto the brain. Tiagabine could then be administered to determine if it is still antiepileptic. Alternatively I could use an *in vitro* model of epileptogenesis in a brain slice, and apply tiagabine with and without the $GABA_B$ antagonist. In this kind of model it is easier to get high concentrations of drug to the tissue, without concerns about pharmacokinetics, blood-brain barrier and so on.

I discussed in chapter 7 the possible reasons for the discrepancy between an 18% reduction in binding and total loss of depression. One possible problem was that

some of the binding detected in *stratum lucidum* will not be on mossy fibres. In order to measure definitively $GABA_B$ receptors on mossy fibre terminals, I could use immunogold labelling of $GABA_B$ receptors and identify the terminals by using the modified Timm's stain. Immunogold labelling has already been used to label $GABA_B$ receptors (Gonchar et al, 2001) and so this approach is feasible using currently available techniques.

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Development/Plasticity/Repair

Plasticity of $GABA_B$ Receptor-Mediated Heterosynaptic Interactions at Mossy Fibers after Status Epilepticus

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Several neurotransmitters, including GABA acting at presynaptic $GABA_B$ receptors, modulate glutamate release at synapses between hippocampal mossy fibers and CA3 pyramidal neurons. This phenomenon gates excitation of the hippocampus and may therefore prevent limbic seizure propagation. Here we report that status epilepticus, triggered by either perforant path stimulation or pilocarpine administration, was followed 24 hr later by a loss of $GABA_B$ receptor-mediated heterosynaptic depression among populations of mossy fibers. This was accompanied by a decrease in the sensitivity of mossy fiber transmission to the exogenous $GABA_B$ receptor agonist baclofen. Autoradiography revealed a reduction in $GABA_B$ receptor binding in the stratum lucidum after status epilepticus. Failure of $GABA_B$ receptor-mediated modulation of mossy fiber transmission at mossy fibers may contribute to the development of spontaneous seizures after status epilepticus.

Key words: epilepsy; $GABA_B$ receptor; status epilepticus; mossy fibers; CA3; seizures

Introduction

Hippocampal mossy fibers represent a major input from dentate granule cells to the hippocampal CA3 field. They exhibit several forms of presynaptic modulation of transmitter release, including marked short-term (Salin et al., 1996) and long-term (Harris and Cotman, 1986) use-dependent plasticity. They are sensitive to several neurotransmitters that depress transmitter release, including glutamate (Kamiya et al., 1996), GABA (Min et al., 1998; Vogt and Nicoll, 1999), and peptides (Weisskopf et al., 1993) acting at metabotropic receptors. Mossy fiber transmission may be under such profound modulation because hippocampal principal cells are highly vulnerable to excitotoxicity (Meldrum, 1993). Nevertheless, these modulatory mechanisms can break down: excessive activity in the dentate gyrus can spread into the hippocampus and can result in neuronal loss that resembles that seen with kainate administration (Sloviter, 1991).

Failure of modulation of mossy fiber transmission may also contribute to the delayed development of spontaneous seizures (epileptogenesis) after an insult. An important example of such a phenomenon is the occurrence of spontaneous seizures after an episode of status epilepticus (SE), defined as seizure activity lasting >30 min (Shorvon, 1994). SE results in acute changes in transmission in the dentate gyrus that could contribute to the maintenance of ongoing seizure activity and the later occurrence of spontaneous seizures (Lothman et al., 1990). Much attention has been given to reductions in inhibition in the dentate gyrus after SE (Sloviter, 1987; Hellier et al., 1999; Doherty and Dingledine, 2001; Kobayashi and Buckmaster, 2003). However, changes also occur in the modulation of excitatory transmission at mossy fiber → CA3 synapses (Goussakov et al., 2000).

We have focused on a form of modulation of mossy fiber → CA3 transmission that reflects the release of endogenous neurotransmitters from neighboring mossy fibers. In acute slices from guinea pigs, a train of stimuli delivered to one group of mossy fibers depresses transmission mediated by another population of mossy fibers. This "heterosynaptic depression" can be mediated both by group II metabotropic glutamate receptors and by $GABA_B$ receptors (Min et al., 1998; Vogt and Nicoll, 1999). The source of GABA that contributes to heterosynaptic depression is unclear. It could originate from interneurons, but another possible source is mossy fibers themselves, which, in addition to glutamate, contain GABA (Sloviter et al., 1996). Indirect evidence for mossy fiber GABAergic transmission has been reported in guinea pigs (Walker et al., 2001) and after seizure-like activity in rats (Gutierrez and Heinemann, 2001).

Here we show that $GABA_B$ receptor-mediated heterosynaptic depression is present at the normal rat mossy fiber synapse, but it is significantly reduced 24 hr after SE in two rodent models. The loss of heterosynaptic depression was seen despite an increase in immunogold staining for mossy fiber GABA. However, it was accompanied by a reduction in the sensitivity of mossy fiber transmission to a $GABA_B$ receptor agonist and by a loss of $GABA_B$ receptor binding revealed by autoradiography. Downregulation of presynaptic $GABA_B$ receptors may play an important role in

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the maintenance of seizure activity during SE and in the development of epilepsy.

Materials and Methods

Epilepsy models

All animal procedures followed the Animal (Scientific Procedures) Act of 1986.

Pilocarpine model. Limbic status epilepticus was induced in adult male Sprague Dawley rats (270–330 gm) by injection of the muscarinic agonist pilocarpine (310–340 mg/kg, i.p.) (Turski et al., 1989). To lessen peripheral cholinergic effects, scopolamine methyl nitrate (1 mg/kg, i.p.) was administered 30 min before and 30 min after pilocarpine. The onset of SE was defined as the appearance of stage 3 (Racine, 1972) seizures followed by continuous clinically detectable seizure activity. Clinically overt SE was terminated after 90–120 min by injection of diazepam (10 mg/kg, i.p.).

Perforant path stimulation model. This method has been described in detail previously (Walker et al., 1999). In brief, male Sprague Dawley rats (270–330 gm) were anesthetized with 1–2% halothane in O_2. An earth electrode was positioned subcutaneously, and a monopolar recording electrode was implanted stereotaxically into the right hippocampus (coordinates, 2.5 mm lateral and 4 mm caudal from bregma). A bipolar stimulating electrode was implanted in the right hemisphere and advanced into the angular bundle (coordinates, 4.4 mm lateral and 8.1 mm caudal from bregma) to stimulate the perforant path. The depths of the electrodes were adjusted to maximize the slope of the dentate granule cell field potential (Walker et al., 1999). The electrodes were held in place with dental acrylic and skull screws. The animals were allowed to recover from anesthesia. Seven days later, the perforant path was electrically stimulated with 2–3 mA 50–150 μsec monopolar pulses at 20 Hz for 2 hr; this induced self-sustaining SE that was terminated after 3 hr with propofol (50 mg/kg, i.p.).

Animals were killed 24 hr or 3 weeks after SE with an overdose of pentobarbitone (500 mg/kg, i.p).

Electrophysiology

Transverse hippocampal slices (400 μm thick) were obtained from control rats and rats after SE and were stored in an interface chamber for at least 1 hr before transfer to a submersion recording chamber. The storage and perfusion solution contained (in mM): 119 NaCl, 2.5 KCl, 4 $MgSO_4$, 4 $CaCl_2$, 26.2 $NaHCO_3$, 1 NaH_2PO_4, and 11 glucose and was gassed with 95% O_2 and 5% CO_2 (23–25°C). Field EPSPs (fEPSPs) were recorded using glass microelectrodes (resistance, ~1 MΩ) filled with the perfusion solution, positioned in the stratum lucidum, the mossy fiber termination zone. Two bipolar stainless steel stimulating electrodes were positioned in the dentate granule cell layer, and 0.5–1 mA pulses (80 μsec duration) were applied with constant current stimulators. The electrode positions and stimulus intensities were adjusted until mossy fiber fEPSPs of maximal amplitude were recorded. Slices were discarded if mossy fiber fEPSPs could not be elicited (see Results), probably reflecting noncongruence of the slicing plane and the plane containing the mossy fibers, which run in a lamellar pattern from the dentate gyrus. This occurred with similar frequency in control and post-SE animals.

Naloxone (10 μM) was present in all two-pathway experiments to avoid interference from opioid receptor-mediated heterosynaptic depression (Weisskopf et al., 1993). Sensitivity to the group II metabotropic glutamate receptor agonist (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)-glycine (DCG-IV, 1 μM) was used in all experiments to verify that fEPSPs were mediated by mossy fiber synapses (Kamiya et al., 1996). The following drugs were used to block $GABA_B$, AMPA/kainate, and group II metabotropic glutamate receptors (mGluRs); CGP52432 (5 μM), SCH50911 (20 μM), 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline (NBQX) (50 μM), and LY341495 (500 nM). NO711 (20 μM) was used to block GABA uptake. The $GABA_B$ receptor agonist baclofen (0.1–10 μM) was used to determine $GABA_B$ receptor sensitivity. Drugs were obtained from (Tocris Cookson, Bristol, UK), except for DCG-IV and NO711 (Sigma, St. Louis, MO).

Immunogold labeling for GABA

For electron microscopic postembedding immunogold labeling, ultrathin brain sections were obtained from adult male Sprague Dawley rats (three controls and two after SE, induced by pilocarpine as described above). Ultrathin sections (50 nm) were cut with a Reichert (Depew, NY) Ultracut and collected on pioloform-coated single-slot nickel grids. Grids were then mounted in a grid support plate, soaked in phosphate buffer (PB) for 30 min, and preincubated in an incubation medium (IM) consisting of PB with 1% bovine serum albumin (A4503; Sigma) and 5% fetal calf serum for 30 min at room temperature. Sections were then incubated with a rabbit anti-GABA antibody (1:4000 in IM; A2052; Sigma) overnight at 4°C. After thorough washing (four times for 10 min in PB) and preincubation in IM (30 min), the secondary antibody (goat anti-rabbit IgG coupled to 10 nm gold particles; Sigma) was applied at a dilution of 1:100 in IM for 4 hr at 37°C. Preparations were washed subsequently in PB (five times for 10 min) before final rinsing in double-distilled water. The sections were contrasted with uranyl acetate (4 min) and Reynold's lead citrate (50 sec) according to standard electron microscopic methods. Preparations were examined using a Philips (Eindhoven, The Netherlands) 201C electron microscope. A control preparation from which the primary antibody was omitted showed no immunolabeling.

Autoradiography

Brains removed from control or post-SE rats were rapidly frozen in isopentane cooled in liquid nitrogen and stored at −70°C. Sections (12 μm thick) were cut on a cryostat (2800 Frigocut; Reichert), thaw-mounted onto Superfrost Plus slides (75 × 25 × 1.0 mm; BDH Chemicals, Poole, UK), and then stored at −20°C until used. On the day of binding experiments, sections were left to equilibrate to room temperature. Slides were then incubated in 50 mM Tris buffer containing 2.5 mM $CaCl_2$, pH 7.4, for 20 min before incubating in fresh buffer for 60 min. They were then dried in room air. Each section was then incubated for 60 min and subsequently dried in [^{3}H]CGP62349 (at concentrations of 0.5, 0.75, 1, 2, 4, and 8 nM) to determine total binding. [^{3}H]CGP62349 at the same concentrations plus CGP54626A (10 μM) was used for incubation and then dried to determine nonspecific binding. The slides were then washed in buffer two times for 1 min and briefly rinsed in distilled water to remove buffer salts. The slides were dried in room air and then apposed to film (Hyperfilm-3H; Amersham Biosciences, Arlington Heights, IL). The films were developed 28 d after exposure to the slides. Quantification of receptor autoradiography was achieved by film densitometry using an image analysis system (Microcomputer Imaging Device; Imaging Research Inc., St. Catharines, Ontario, Canada), and optical density was converted to femtomoles per milligram of bound ligand. Total binding in the stratum lucidum was assessed in four to eight sections per concentration of [^{3}H]CGP62349 for each animal. Binding parameters, receptor density (B_{max}), and affinity (K_D) were determined by the use of the Langmuir equation in Prism PC software (Graph Pad, San Diego, CA). [^{3}H]CGP62349 was a gift from Dr. Wolfgang Froestl (Novartis, Basel, Switzerland).

Data are expressed as mean ± SEM. The baclofen data were analyzed using a best least squares fit to $I_{max}/(1 + IC_{50}/[\text{baclofen}])$, where I_{max} is the maximal inhibition, and the IC_{50} is the concentration of baclofen that results in half-maximal inhibition (KyPlot; KyensLab Inc., Tokyo, Japan). Results were compared using two-tailed paired or unpaired Student's *t* test, as appropriate.

Results

$GABA_B$ receptors mediate heterosynaptic depression in rats

Because the role of $GABA_B$ receptors in heterosynaptic depression has hitherto only been studied in juvenile guinea pigs, we first asked whether it could be induced at the mossy fiber → CA3 synapse in acute hippocampal slices from adult control rats. All data were obtained with the opioid receptor antagonist naloxone (10 μM) in the perfusion solution to remove any confounding effect of presynaptic opioid receptors (Weisskopf et al., 1993). The recording solution normally contained 4 mM Ca^{2+} and 4 mM Mg^{2+} to reduce the likelihood of epileptiform bursting. We first verified that the two stimulating electrodes in stratum granulosum evoked fEPSPs recorded in the stratum lucidum with prop-

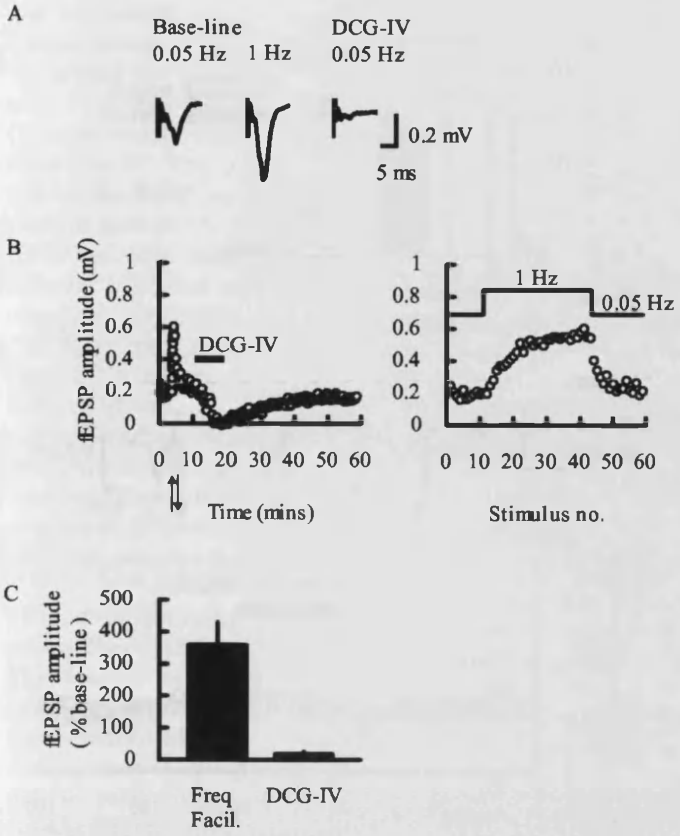

Figure 1. Mossy fiber fEPSPs were identified by demonstrating marked frequency facilitation (Freq Facil.) and sensitivity to the group II metabotropic glutamate receptor agonist DCG-IV. *A*, fEPSPs (traces are averages of 5 trials, taken from experiment shown in *B*) recorded in the stratum lucidum while stimulating the stratum granulosum of the dentate gyrus at baseline frequencies of 0.05, 1, and 0.05 Hz in the presence of the group II metabotropic glutamate receptor DCG-IV (1 μM). *B*, Left graph, Example of an experiment demonstrating the identification of mossy fiber fEPSPs by frequency facilitation (arrows, change in stimulation frequency from 0.05 to 1 Hz) and DCG-IV sensitivity. Right graph, Same experiment showing the effect of an increase in frequency plotted against stimulus number. *C*, Summary data ($n = 8$). The fEPSP amplitude increased to at least 200% of baseline (20th/1st response) when the stimulus frequency was increased from 0.05 to 1 Hz, and the mGluR agonist DCG-IV (1 μM) decreased fEPSP amplitude to <30% of baseline.

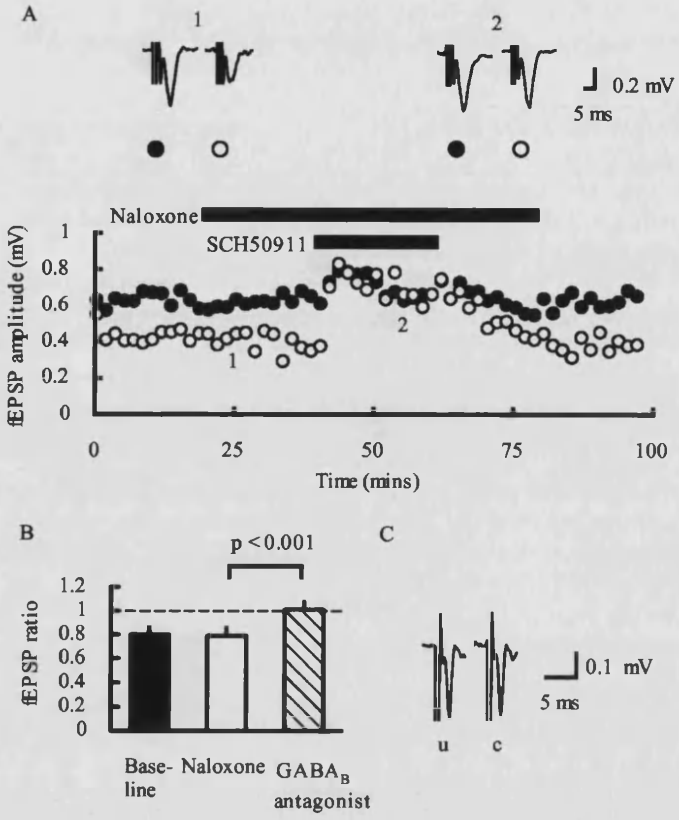

Figure 2. Heterosynaptic depression evoked in control slices is mediated by $GABA_B$ receptors. A conditioning train was applied to the conditioning pathway preceding every 10th stimulus delivered to the test pathway. *A*, Example of one experiment from a control slice. Heterosynaptic depression was observed as a reduction in amplitude of the fEPSPs that were preceded by a train (open circles) compared with fEPSPs not preceded by a train (each filled circle represents the average of the 9 unconditioned fEPSPs in each cycle). The opioid antagonist naloxone (10 μM) had no effect on the magnitude of depression. The $GABA_B$ receptor antagonist SCH50911 (20 μM) abolished the depression. fEPSPs (averages of 5 trials each) are shown in the absence (1) and presence (2) of SCH50911. *B*, Summary of the ratio of conditioned/unconditioned fEPSP amplitudes in 8 control slices showing the abolition of heterosynaptic depression by blocking $GABA_B$ receptors. *C*, Example of presynaptic fiber volleys from one experiment showing that the conditioned presynaptic fiber volley [preceded by a conditioning train (c)] is not different in amplitude or shape from the unconditioned fiber volley [not preceded by a train (u)]. Traces are averages of responses in the presence of 50 μM NBQX from two cycles of nine unconditioned stimuli and one conditioned stimulus.

erties consistent with mossy fibers. fEPSPs showed marked short-term frequency-dependent facilitation (increase in fEPSP amplitude more than twice baseline) when the stimulation frequency was increased from 0.05 to 1 Hz (Fig. 1*A–C*). In all experiments, we confirmed that they showed high sensitivity to the group II mGluR agonist DCG-IV (1 μM), characteristic of mossy fiber synapses (Kamiya et al., 1996) (Fig. 1A,B,C).

In addition, we verified that the two pathways were separate by confirming that the fEPSP elicited in one pathway was not facilitated by a preceding stimulus to the other pathway (cross-facilitation was 4 ± 3%; $n = 6$).

At the end of each experiment, we applied the AMPA/kainate receptor antagonist NBQX (50 μM) to record the remaining presynaptic fiber volley, which was then subtracted from the fEPSP before analysis of the results. A high concentration of NBQX was used to ensure that all AMPA and kainate receptors were blocked (Bortolotto et al., 1999).

Once two separate mossy fiber pathways had been identified, we used the following stimulation protocol to induce heterosynaptic depression. Single stimuli were applied to the "test" pathway at a frequency of 0.05 Hz. Three hundred milliseconds before every 10th stimulus, a train of five stimuli at 50 Hz was applied to the "conditioning" pathway. The magnitude of heterosynaptic depression was calculated by comparing the amplitude of every 10th fEPSP recorded [conditioned fEPSP ($fEPSP_C$)] to the average amplitude of the nine preceding fEPSPs [unconditioned fEPSP ($fEPSP_U$)]. The fEPSP ratio was calculated as $fEPSP_C/fEPSP_U$.

Heterosynaptic depression, expressed as (1 − fEPSP ratio) × 100%, was observed in all slices from adult control rats (21 ± 7% depression; $n = 8$ slices from different animals) (Fig. 2*A,B*). Bath perfusion of $GABA_B$ receptor antagonists (20 μM SCH50911; $n = 6$; or 5 μM CGP52432; $n = 2$) completely abolished heterosynaptic depression in all cases (0 ± 6% depression; $p < 0.001$ for comparison with baseline) (Fig. 2*A,B*). Thus, in slices from control adult rats, heterosynaptic depression evoked with this protocol was entirely mediated by $GABA_B$ receptors, with no detectable contribution from mGluRs (Min et al., 1998; Vogt and Nicoll, 1999).

Blocking $GABA_B$ receptors also caused a small increase in the size of the unconditioned fEPSPs in two of eight control slices (Fig. 2*A*). This did not reach significance in either control or

post-SE slices when all experiments were included ($p = 0.45$; paired t test). Because heterosynaptic interactions mediated by spillover of the transmitter are temperature-dependent (Asztely et al., 1997; Mitchell and Silver, 2000), we also measured heterosynaptic depression at 34°C, using the same stimulation protocol. $GABA_B$ receptor-mediated heterosynaptic depression was identical to that recorded at room temperature (21 ± 8% depression; $n = 3$ slices from different animals; $p = 0.9$; unpaired t test, when compared with depression at room temperature).

To verify that this phenomenon is present at more physiological concentrations of divalent cations, we repeated the experiments with a perfusion solution containing 2.5 mM Ca^{2+} and 1.3 mM Mg^{2+}. In two slices, this yielded heterosynaptic depression of 33 ± 4%, which was reduced to 4 ± 2% with $GABA_B$ receptor antagonists.

Although $GABA_B$ receptors profoundly depress transmitter release, a potentially confounding effect is efflux of K^+ from neurons, which could alter axon recruitment, action potential propagation, or both. We therefore compared the presynaptic fiber volleys (recorded in the presence of NBQX to abolish the fEPSP) with and without the 50 Hz train in the conditioning pathway. Neither the amplitude ($p = 0.69$; paired t test; $n = 8$) nor the shape of the fiber volley was significantly affected (Fig. 2*D*), implying that heterosynaptic depression is not mediated by an increase in extracellular K^+.

SE abolishes $GABA_B$ receptor-mediated heterosynaptic depression

We interleaved the experiments described above with identical experiments performed on hippocampal slices taken from rats 24 hr after pilocarpine-induced SE. We used the same criteria to identify two separate mossy fiber pathways (cross-facilitation, 0 ± 6%; $n = 11$).

The degree of frequency-dependent facilitation was not significantly different from that measured in control slices (post-SE, 232 ± 25% of baseline, $n = 12$; control, 358 ± 67%, $n = 8$; measured as 20th fEPSP/1st fEPSP amplitude when stimulation was increased from 0.05 to 1Hz; $p = 0.1$) (Fig. 3*A*). The nonsignificant trend for less frequency facilitation in the post-SE animals is consistent with the findings of Goussakov et al. (2000), who showed a significant loss of facilitation after kainic acid-induced SE. The more marked changes observed by Goussakov et al. (2000) may be attributable to differences in stimuli to induce SE (kainic acid vs pilocarpine), stimulation frequency (100 instead of 1 Hz), a longer interval from SE to killing of the animals (several weeks instead of 24 hr), or a combination thereof.

In post-SE slices, DCG-IV perfusion reduced the fEPSPs to 22 ± 5% of baseline. This depression was also indistinguishable from that seen in control slices (19 ± 8%; $p = 0.8$) (Fig. 3*A*).

In contrast to control animals, heterosynaptic depression was not detected after pilocarpine-induced SE (1 ± 4% depression; $n = 12$; $p = 0.75$; paired t test comparing conditioned with unconditioned fEPSPs) (Fig. 3*B*,*C*). Thus, despite the apparently normal short-term plasticity and mGluR sensitivity of mossy fiber synapses, they were unaffected by trains of stimuli delivered to neighboring axons.

These results do not distinguish between a generic effect of limbic seizures and a specific result of the chemoconvulsant used to evoke SE. We therefore asked whether a different model of SE, which does not involve the use of an exogenous convulsant, also interfered with $GABA_B$ receptor-mediated modulation of mossy fiber transmission. We examined hippocampal slices taken from six animals 24 hr after SE evoked by perforant path stimulation

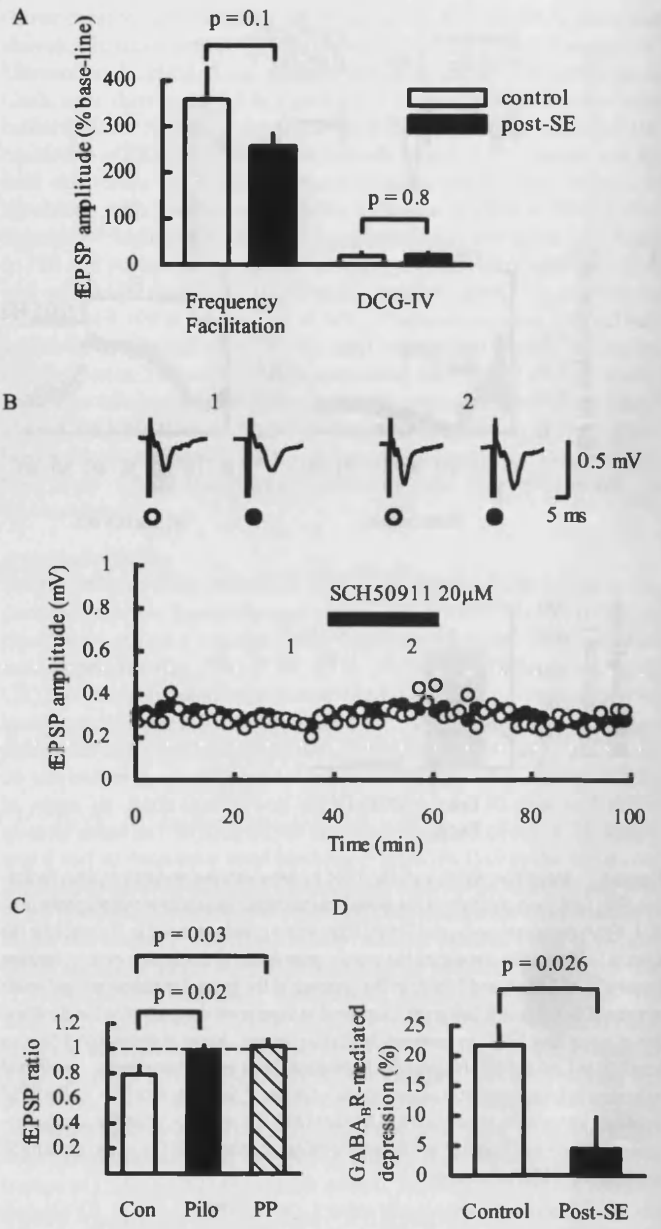

Figure 3. $GABA_B$ receptor-mediated heterosynaptic depression was absent after SE. *A*, Mossy fiber fEPSPs in slices after pilocarpine-induced SE ($n = 12$) showed frequency facilitation and DCG-IV sensitivity, which was not significantly different from that of control slices. *B*, Heterosynaptic depression was absent after pilocarpine-induced SE: example of one experiment. fEPSPs are shown in the absence and presence of SCH50911 (each trace is the average of 5 trials; filled circles, conditioned; open circles, unconditioned). The $GABA_B$ receptor antagonist SCH50911 had no significant effect. *C*, There was no significant heterosynaptic depression in animals after pilocarpine-induced (Pilo) or perforant path (PP) stimulation-induced SE in contrast to the marked depression seen in control (Con) animals. *D*, Size of $GABA_B$ receptor-mediated heterosynaptic depression, estimated from the effect of the $GABA_B$ antagonists SCH50911 and CGP52432 on the fEPSP ratio (SE: both models combined; $n = 18$).

(see Materials and Methods). Heterosynaptic depression was again absent in slices from all six animals (−2 ± 5% depression; $n = 6$) (Fig. 3*C*). We also examined slices from two animals treated with pilocarpine that had not developed SE. Both animals received diazepam injections identical to those administered to the animals that experienced SE. Heterosynaptic depression was observed in slices from these animals (45 ± 4% depression). Thus, we conclude that loss of heterosynaptic depression is related to SE per se rather than to the stimulus used to evoke it.

To test whether the loss of heterosynaptic depression after SE

was attributable to the emergence of a compensatory heterosynaptic facilitation, we applied the $GABA_B$ receptor antagonists SCH50911 (20 μM) and CGP52432 (5μM) during the two-pathway protocol in slices after SE. This had no significant effect on the fEPSP ratio in slices from the post-SE animals (5 $\pm$ 4% change in the fEPSP ratio; $n = 18$). This was significantly different from that of controls (22 $\pm$ 3% change in the fEPSP ratio; $n = 8$) when $GABA_B$ receptor antagonists were applied ($p = 0.026$; unpaired t test for difference between SE and control animals). This result implies that loss of heterosynaptic depression after SE was not attributable to the emergence of a compensatory heterosynaptic facilitation but because of failure of $GABA_B$ receptor-mediated depression.

SE is accompanied by an increase in mossy fiber GABA

The loss of heterosynaptic depression could contribute to the persistence of seizure activity during SE, to the subsequent development of epilepsy, or to both. The precise relationship of the failure of $GABA_B$ receptor-mediated heterosynaptic depression to these phenomena is beyond the scope of the present study. Instead, we asked what cellular alterations could underlie this result.

There are two main sources of GABA that could mediate this heterosynaptic depression. First, the GABA could originate from mossy fibers because there is circumstantial evidence that mossy fibers themselves can release GABA (see Introduction). Second, interneurons recruited by the mossy fibers could release GABA that spills into the extracellular space. From the first hypothesis, a possible explanation for loss of $GABA_B$ receptor-mediated heterosynaptic depression is that there is a reduction in the releasable GABA in mossy fibers after SE. This is, however, difficult to reconcile with reports that immunolabeling for GABA and glutamic acid decarboxylase increases after seizure-like activity (Sloviter et al., 1996) and that this is accompanied by the emergence of a monosynaptic GABAergic signal (Gutierrez and Heinemann, 2001). Nevertheless, much of the evidence for increased mossy fiber GABA is difficult to relate directly to the present study because of different seizure models or because different criteria were used to identify mossy fibers. Indeed, most reports have relied on light microscopy, which does not ensure that the epitope is localized to mossy fiber terminals. We therefore applied immunogold electron microscopy to detect GABA-like immunoreactivity in mossy fiber terminals.

Figure 4 shows examples of electron micrographs taken from the stratum lucidum. Mossy fiber terminals were identified by their large size, multiple active zones, and numerous vesicle profiles. In addition, we also used a modified Timm's reaction in one control animal and one post-SE animal to confirm the identity of the mossy fiber terminals on the basis of their high zinc content (Seress and Gallyas, 2000). We did not use these animals for quantitative analysis because a different fixation method was used. Immunogold particles in mossy terminals of control animals (Fig. 4*C*,*D*) were present at a density of 12 $\pm$ 1/μm^2 (56 terminals from two animals). This was significantly greater than the background density of 4 $\pm$ 1/μm^2 ($p < 0.001$). We repeated these measurements in two post-SE animals (Fig. 4*A*,*B*). The GABA immunogold particle density in mossy fiber terminals was doubled 24 hr after SE induced by pilocarpine to 24 $\pm$ 2/μm^2 (45 terminals from two animals; $p < 0.001$ compared with controls). Thus, the immunolabeling, although confirming more indirect evidence for GABA in mossy fibers, lends no support to the hypothesis that the amount available to be released is decreased after SE. On the contrary, there is more GABA in mossy fibers after SE, in keeping with previous reports that seizure-like activity is followed by the emergence of a monosynaptic GABAergic signal in CA3 pyramidal neurons (Gutierrez and Heinemann, 2001). Thus, to explain the loss of heterosynaptic depression after SE, it is necessary to postulate either that (1) the source of GABA mediating heterosynaptic depression is not mossy fibers but interneurons, or (2) a change downstream of GABA release is responsible.

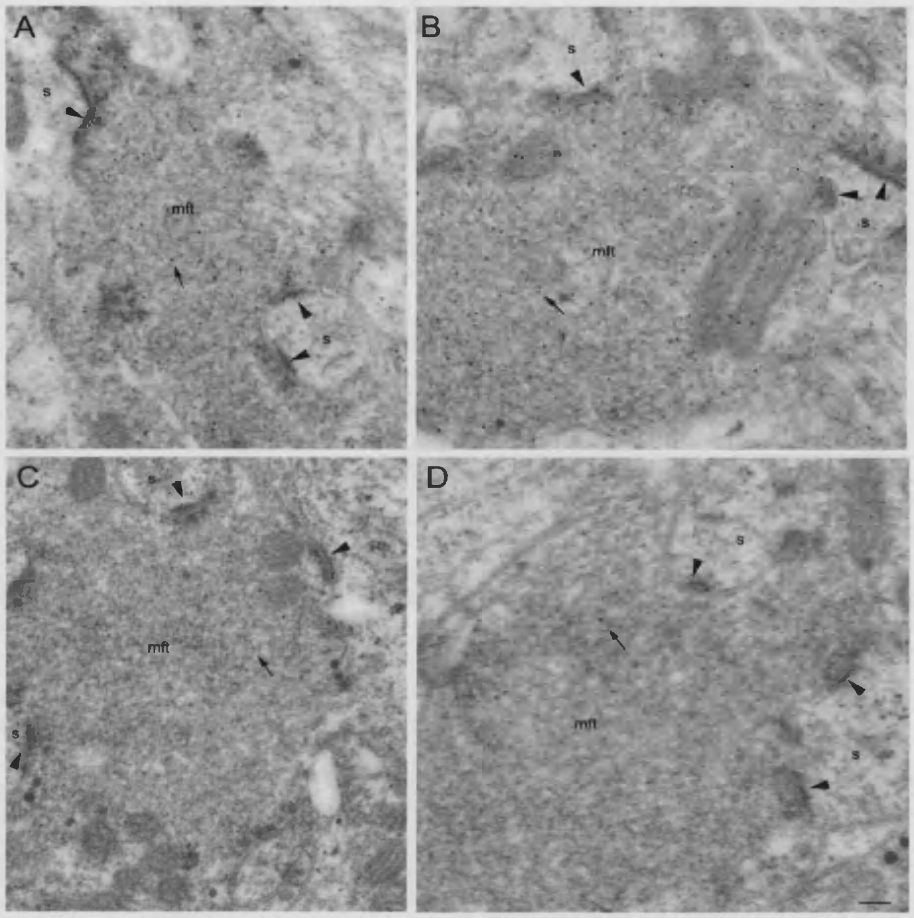

Figure 4. Sections through GABA immunogold-labeled mossy fiber terminals in SE (*A*, *B*) and control (*C*, *D*) animals. Tissue from animals after SE have significantly higher density of immunogold particles (arrows). Arrowheads, Synaptic sites; mft, mossy fiber terminal; s, spine. Scale bar: *A*, *B*, 100 nm; *C*, 120 nm; *D*, 60 nm.

Heterosynaptic depression is not restored by blocking mGluRs or GABA uptake

If the GABA that mediates heterosynaptic depression is released from interneurons, a possible explanation for the effect of SE is impaired recruitment of interneurons, which has been described in several models of epilepsy (Sloviter, 1991; Lothman et al., 1996; Doherty and Dingledine, 2001). Reduced recruitment of inter-

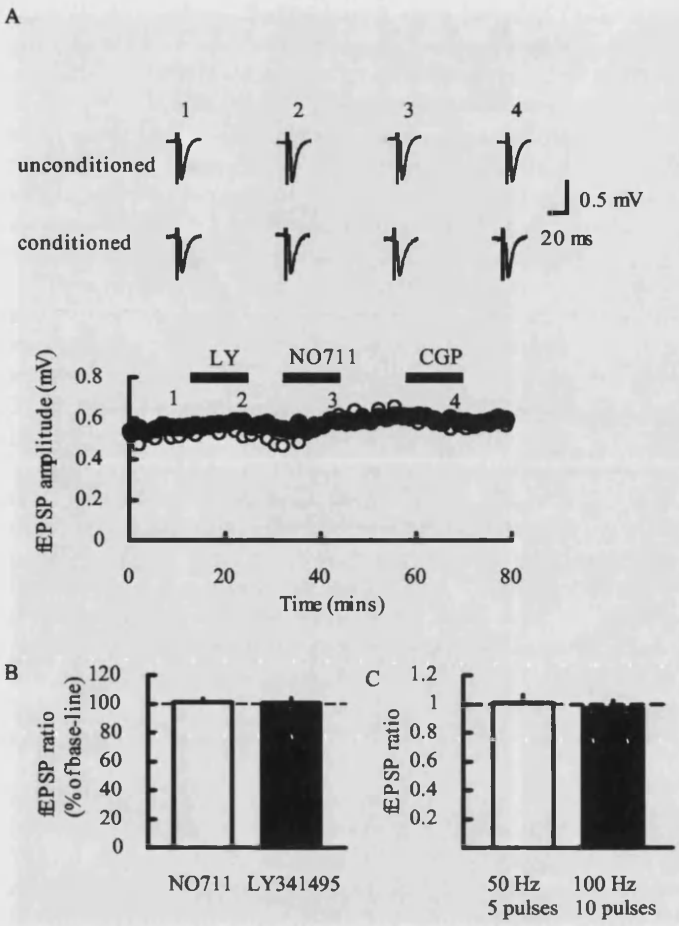

Figure 5. Heterosynaptic depression could not be rescued after SE by blocking group II metabotropic glutamate receptors by blocking GABA uptake or by increasing the number of conditioning stimuli. *A*, Example of one experiment. The group II metabotropic glutamate receptor antagonist LY341495 (LY; 500 nM) failed to restore heterosynaptic depression in a slice obtained after pilocarpine-induced SE. Application of the GABA transporter GAT1 blocker NO711 (20 μM) also failed to affect the fEPSP ratio. Sample traces, fEPSPs in control conditions and in the presence of LY34195, NO711, and CGP52432 (each trace is the average of 5 trials). *B*, Summary of effects of NO711 ($n = 4$) and LY341495 ($n = 5$) on the fEPSP ratio in animals after pilocarpine-induced SE. *C*, A 100 Hz train did not rescue heterosynaptic depression in slices after SE. The fEPSP ratio did not change significantly when the train was increased from 50 to 100 Hz ($p = 0.4$; paired *t* test).

neurons in the dentate hilus after SE has been proposed to be mediated by enhanced activity of group II mGluRs (Doherty and Dingledine, 2001). We therefore asked whether heterosynaptic depression could be rescued in slices from post-status animals by blocking group II mGluRs. We applied the group II mGluR antagonist LY341495 (500 nM) during the two-pathway experiment to slices from animals 24 hr after pilocarpine-induced SE. This had no significant effect on the magnitude of heterosynaptic depression ($p = 0.18$; paired *t* test; $n = 5$) (Fig. 5*A*,*B*). This concentration of LY341495 was sufficient to antagonize the effect of the group II mGluR agonist DCG-IV on mossy fiber transmission completely ($n = 3$; data not shown). We then asked whether changing the stimulation protocol to enhance the recruitment of interneurons could rescue depression. We applied the same stimulation protocol of five pulses at 50 Hz and then in the same slices increased the train to 10 pulses at 100 Hz (we tested this protocol in slices from four rats, two of which had pilocarpine-induced status epilepticus and two of which had perforant path stimulation-induced status epilepticus). Heterosynaptic depression was not rescued. The fEPSP ratio was 1 ± 0.1 during the 50 Hz protocol and 0.97 ± 0.05 during the 100 Hz protocol. The fEPSP ratio was not significantly different between 50 and 100 Hz trains (Fig. 5*C*). This provides no evidence for the hypothesis that impaired recruitment of interneurons accounts for the loss of heterosynaptic depression after SE.

Another possible explanation is that GABA uptake is enhanced. Altered GABA transport has been implicated in the pathogenesis of epilepsy (Patrylo et al., 2001). The antiepileptic drug tiagabine is a blocker of neuronal GABA transporter 1 (GAT1) (Andersen et al., 1993). A decrease in immunostaining for GAT1 has been reported in the sensorimotor cortex (Silva et al., 2002) and the hippocampus (Andre et al., 2001). We therefore attempted to rescue heterosynaptic depression by interfering with the major hippocampal GABA transporter GAT1 with the selective blocker NO711. We applied the same two-pathway protocol as described previously in slices from rats 24 hr after pilocarpine-induced SE. Bath application of 20 μM NO711 (which is sufficient to evoke a tonic $GABA_A$ receptor-mediated current in pyramidal neurons; Semyanov et al., 2003) failed to restore heterosynaptic depression ($n = 4$; $p = 0.88$; paired *t* test) (Fig. 5*A*,*B*). These results thus lend no support to the hypothesis that heterosynaptic depression was lost because of increased uptake.

Status epilepticus alters $GABA_B$ receptors on mossy fibers

A further explanation for loss of heterosynaptic depression is an alteration in the target $GABA_B$ receptors. Altered expression of $GABA_B$ receptors in CA3 pyramidal neurons has been reported in human temporal lobe epilepsy patients (Princivalle et al., 2002), and decreased presynaptic $GABA_B$ receptor activity has been proposed to underlie increased inhibitory activity in the dentate gyrus during epileptogenesis (Haas et al., 1996). We therefore asked whether there was loss of $GABA_B$ receptor function after SE. We stimulated a single mossy fiber pathway at 0.05 Hz in acute hippocampal slices taken either from control animals or from animals after pilocarpine-induced SE and measured the sensitivity of the fEPSP to increasing concentrations of the $GABA_B$ receptor agonist baclofen. Baclofen was less potent in reducing the fEPSP in the postpilocarpine SE slices compared with its effect in control slices (controls, IC_{50}, 0.47 ± 0.14 μM, $n = 6$; SE, IC_{50}, 1.17 ± 0.27 μM, $n = 7$; $p < 0.05$ for difference) (Fig. 6). There was no significant difference in the I_{max} by baclofen (controls, I_{max}, 0.95 ± 0.05, $n = 6$; SE, I_{max}, 0.87 ± 0.06, $n = 7$; $p = 0.31$ for difference). Loss of $GABA_B$ receptor function may contribute to the development of self-sustaining seizures in SE. This could lead to increased excitability in the hippocampus and could play a role in the later development of spontaneous seizures. We therefore asked whether this change in $GABA_B$ receptor function was persistent. We treated six animals with pilocarpine to induce 90 min of SE. These were killed 3 weeks later when they were exhibiting spontaneous seizures (all animals had several limbic seizures per day). We recorded fEPSPs in the stratum lucidum while stimulating in the dentate gyrus as above. Although the increase in IC_{50} for baclofen did not reach significance when compared with that in controls, the I_{max} was significantly reduced (0.74 ± 0.05; $p = 0.007$; unpaired *t* test) (Fig. 6*B*).

We then asked whether the reduced potency of baclofen at 24 hr was attributable to a change in the number or functional properties of $GABA_B$ receptors. We used [^{3}H]CGP62349 autoradiography to estimate the density of $GABA_B$ receptors in the stratum lucidum in control and post-pilocarpine-induced SE tissue (24 hr after SE). The radiolabeled antagonist was applied at concentrations of 0.5, 0.75, 1, 2, 4, and 8 nM. The mean K_D for post-SE

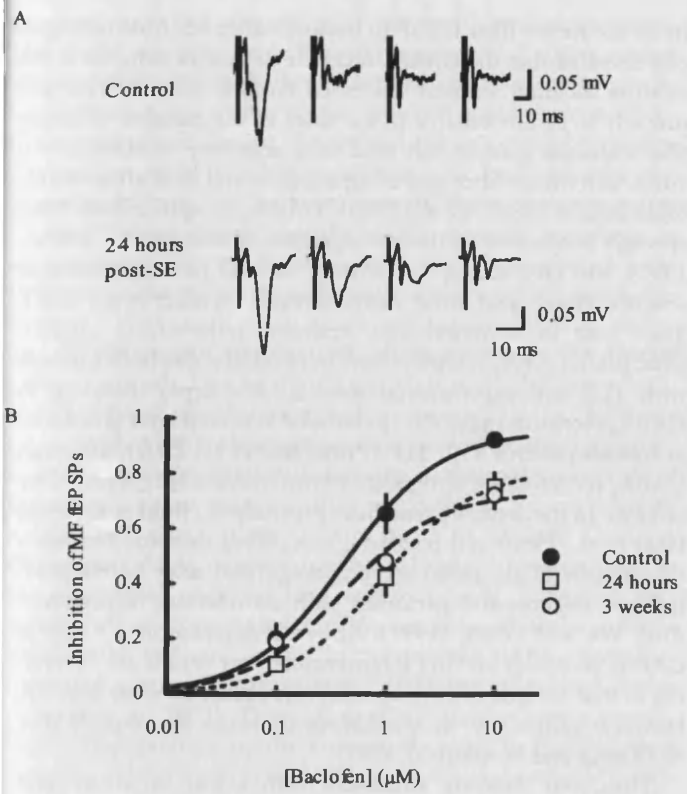

Figure 6. Mossy fiber fEPSPs become less sensitive to the $GABA_B$ receptor agonist baclofen after SE. *A*, fEPSPs (averages of 5 traces) illustrating reduced sensitivity to baclofen 24 hr after SE. Left to right, Traces are shown at baseline and in 0.1, 1, and 10 μM baclofen. *B*, Baclofen has a less potent effect on mossy fiber fEPSPs 24 hr after SE ($p < 0.05$; unpaired *t* test on IC_{50} values; 6 control slices and 7 post-SE slices), and the I_{max} was significantly reduced in slices 3 weeks after SE ($p = 0.007$; $n = 6$).

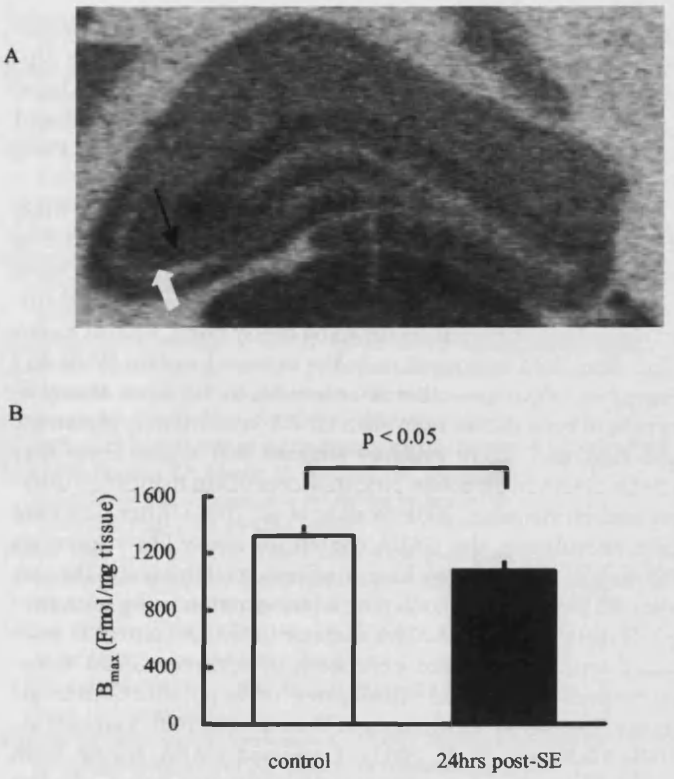

Figure 7. Reduction of binding of [^{3}H]CGP62349 to $GABA_B$ receptors in the stratum lucidum after pilocarpine-induced SE. *A*, Example of an autoradiograph from an animal after pilocarpine-induced SE. We measured binding in the stratum lucidum, (black arrow). The stratum pyramidale of CA3 is marked with a white arrow. *B*, The binding parameter B_{max}, which reflects maximal binding of the radioactive ligand to $GABA_B$ receptors, was significantly lower than that of the control after SE (control, $n = 5$; SE, $n = 4$; $p = 0.033$ for difference).

animals was not significantly different from that for control animals, implying that the affinity of the $GABA_B$ receptors was unchanged (controls, 1.28 ± 0.34 nM, $n = 5$; SE, 1.09 ± 0.10 nM, $n = 4$; $p = 0.67$). However, the mean B_{max} (a measure of $GABA_B$ receptor density) 24 hr after SE was significantly less than in controls (controls, 1322 ± 68 fmol/mg of tissue, $n = 5$; SE, 1080 ± 56 fmol/mg of tissue, $n = 4$; $p < 0.05$ for difference) (Fig. 7). We also observed a nonsignificant trend for the B_{max} to be reduced at 3 weeks (1144 ± 54 fmol/mg of tissue; $n = 4$; $p = 0.09$ for difference from control).

Is the reduction in $GABA_B$ receptor density specific to the presynaptic $GABA_B$ receptors on mossy fiber terminals, or is it a global change that also occurs in the dentate granule cell dendrites? To address this question, we measured binding in the molecular layer of the dentate gyrus. The mean B_{max} and K_D in 24 hr post-SE tissue were not significantly different from those of controls (B_{max}: controls, 925 ± 74 fmol/mg of tissue, $n = 5$; SE, 812 ± 71 fmol/mg of tissue, $n = 4$; $p = 0.29$; K_D: controls, 0.69 ± 0.20 nM; SE, 0.82 ± 0.23 nM; $p = 0.83$).

Thus, the loss of $GABA_B$ receptor-mediated heterosynaptic depression at 24 hr is accompanied by a selective reduction in $GABA_B$ receptor density in the stratum lucidum and an associated reduction in the sensitivity of mossy fiber signaling to $GABA_B$ receptor agonists. The 18% reduction in $GABA_B$ receptor binding in the stratum lucidum is nevertheless modest compared with the complete loss of $GABA_B$ receptor-mediated heterosynaptic depression. Among possible explanations for this discrepancy are nonlinear amplification of the intracellular signaling cascade linking receptors to transmitter release and the fact that, within the stratum lucidum, other cell types also express $GABA_B$ receptors, some of which may be upregulated (see Discussion).

Discussion

We have demonstrated that $GABA_B$ receptor-mediated heterosynaptic depression at the rat mossy fiber synapse is lost after SE, whether this is evoked by pilocarpine or perforant path stimulation. This loss is not attributable to a compensatory heterosynaptic facilitation or to depletion of GABA from mossy fiber terminals. We could not rescue it by applying group II mGluR antagonists or GABA transporter inhibitors. Instead, we find that the sensitivity of mossy fiber synapses to $GABA_B$ receptor agonists diminishes after SE, and this is mirrored by a decrease in $GABA_B$ receptor binding density. Thus, SE results in a loss of $GABA_B$ receptors that is sufficient to impair the detection of physiological levels of their endogenous ligand.

The role of heterosynaptic interactions among mossy fibers in gating excitatory afferent traffic to the hippocampus *in vivo* remains to be determined. Impairment of mutual activity-dependent depression among mossy fibers could contribute to lowering the threshold for seizure spread through mesial temporal structures. These changes could play a role in the development of temporal lobe epilepsy (TLE) and the ancillary damage to principal neurons in the hippocampus. The plasticity revealed in the present study must be seen within the broader context of other changes in inhibition occurring in the hippocampus during epileptogenesis, such as those occurring within the dentate gyrus and in the hippocampus proper. Altered inhibition is observed frequently in both experimental and human epilepsy. Both in-

creases (Haas et al., 1996) and decreases (Sloviter, 1987; Bekenstein and Lothman, 1993; Doherty and Dingledine, 2001) in hippocampal inhibition have been reported. Most studies have, however, concentrated on changes in $GABA_A$ receptor-mediated inhibition (Buhl et al., 1996; Kapur and Macdonald, 1997; Brooks-Kayal et al., 1998).

The loss of heterosynaptic depression observed in this study did not appear to be attributable to decreased GABA release or increased GABA uptake. There are two potential sources of GABA release after the conditioning train: mossy fibers themselves and interneurons recruited by mossy fibers, such as mossy fiber associated interneurons in the stratum lucidum (Vida and Frotscher, 2000) and other interneurons in the hilus. Mossy fibers have been shown to contain GABA in addition to glutamate and zinc, and recent evidence suggests that mossy fibers may release GABA in an action potential-dependent manner (Gutierrez and Heinemann, 2001; Walker et al., 2001). After perforant path stimulation, the GABA content of mossy fibers increases (Sloviter et al., 1996). We have confirmed that this is also the case after SE induced by pilocarpine administration using immunogold labeling for GABA. This increase in GABA content is associated with an increased expression of vesicular GABA transporter and an associated enhancement of the putative GABAergic mossy fiber signal (Gutierrez and Heinemann, 2001; Lamas et al., 2001; Gutierrez et al., 2003). Decreased GABA release from mossy fibers, therefore, cannot explain our observation of a loss of heterosynaptic depression because, if anything, the increased GABA released from mossy fibers should enhance heterosynaptic depression.

Interneurons recruited by mossy fibers are other possible sources of GABA. Loss of interneurons could undoubtedly contribute to a decrease in GABA release and so a decrease in heterosynaptic depression. Although populations of interneurons are lost after SE, interneurons in CA3 are relatively well preserved after lithium- and pilocarpine-induced seizures (Andre et al., 2001). Nevertheless, we cannot exclude interneuronal dysfunction as a contributor to the loss of heterosynaptic depression in our models. There are also other possible mechanisms, including decreased recruitment of interneurons via enhancement of presynaptic group II mGluR depression of mossy fiber transmission (Doherty and Dingledine, 2001). We were, however, unable to rescue heterosynaptic depression by applying a group II mGluR antagonist. This argues against the hypothesis that overactivity of group II mGluRs explains the loss of heterosynaptic depression after SE.

The concentration of GABA detected by mossy fiber terminals depends not only on the amount of GABA released but also on the efficiency of GABA uptake. The loss of heterosynaptic depression could thus be explained by enhanced GABA uptake after SE. Anatomical and functional studies have, however, suggested that GABA uptake is decreased after SE. The predominant GABA transporter GAT1 is downregulated in rodent epilepsy models (Andre et al., 2001), and functional impairment of GABA transport has also been reported in tissue obtained either from patients with mesial temporal sclerosis or from rats after kainic acid-induced SE (Patrylo et al., 2001). Our finding that inhibiting GABA uptake does not lead to enhanced heterosynaptic depression in epileptic animals further argues that increased GABA uptake does not play a part in the loss of heterosynaptic depression.

Finally, we explored the possibility that the target $GABA_B$ receptors are lost or fail to modulate transmitter release by measuring the effect of the $GABA_B$ receptor agonist baclofen on the amplitude of mossy fiber fEPSPs. We observed reduced sensitivity of the mossy fiber fEPSP to baclofen after SE. Autoradiography revealed that the $GABA_B$ receptor density is reduced in the stratum lucidum without decreased binding affinity. This was unlikely to be attributable to a change in the number of mossy fibers because granule cells tend to be relatively resistant to seizures, and mossy fiber sprouting starts several days after excitotoxic insults (Mello et al., 1993). $GABA_B$ receptors have been strongly implicated in the pathogenesis of absences (Liu et al., 1992), and rats lacking the $GABA_{B1}$ subunit have spontaneous absence, clonic, and tonic–clonic seizures (Schuler et al., 2001). Their role in temporal lobe epilepsy, however, is unclear. Changes in $GABA_B$ receptors have been observed in both humans with TLE and experimental models of epilepsy. Binding to $GABA_B$ receptors in the CA3 pyramidal neuronal layer is reduced in human patients with TLE (Princivalle et al., 2002), although $GABA_B$ receptors are upregulated when the data are corrected for cell loss. In the dentate gyrus, both presynaptic (Buhl et al., 1996; Haas et al., 1996) and postsynaptic $GABA_B$ receptor functions (Wasterlain et al., 1996) are downregulated after kainic acid-induced seizures and perforant path stimulation, respectively. Also, Wu and Leung (1997) showed a decrease in efficacy of $GABA_B$ receptors on CA1 interneurons. Our results are surprising in that the loss of $GABA_B$ receptors occurs so soon after SE. However, others have shown that receptors can alter rapidly after SE (Kapur and Macdonald, 1997).

Thus, our data are consistent with a loss of presynaptic $GABA_B$ receptors on mossy fiber terminals. This parallels the recent finding that $GABA_{B1}$ and $GABA_{B2}$ receptor mRNA levels are transiently reduced after kainic acid-induced SE (Furtinger et al., 2003). Loss of presynaptic $GABA_B$ receptors renders mossy fibers relatively resistant to GABA, whether this is released directly from the mossy fibers themselves or from interneurons recruited by mossy fibers, explaining the loss of heterosynaptic depression after SE. A possible weakness of this hypothesis is that there is a quantitative discrepancy between the complete loss of heterosynaptic depression and the ~20% decrease in [^{3}H]CGP62349 binding density. This can be partly explained by the $GABA_B$ receptor binding detected in the stratum lucidum being not exclusively attributable to receptors on presynaptic mossy fiber terminals. Indeed, there are also interneurons present in this area that express $GABA_B$ receptors, and increased expression of $GABA_B$ receptors in these interneurons with seizures (Kokaia and Kokaia, 2001) could mask the loss at mossy fiber terminals. Also, intracellular amplification of metabotropic receptor-mediated actions may lead to a nonlinear relationship between receptor density and sensitivity to GABA spillover.

Could changes in the extracellular space contribute to the loss of heterosynaptic depression? Status epilepticus induces CNS edema, which may last several days (Roch et al., 2002). Cell swelling during excessive neuronal activity can result in a 30% decrease in the volume of the extracellular space (Lux et al., 1986) and could change concentrations of extracellular ions and transmitters. Although an experimental investigation of this possibility was beyond the scope of this study, a decrease in the extracellular space might be expected to increase the extracellular GABA concentration after exocytosis and, therefore, to increase $GABA_B$ receptor-mediated heterosynaptic depression. However, if this is accompanied by increased tortuosity of the extracellular space, the diffusional path for GABA molecules from their release sites to the receptors could be increased, with the opposite effect on heterosynaptic depression.

Loss of heterosynaptic depression may lead to propagation of

excessive excitatory afferent traffic into the hippocampus proper. This could also play a role in epileptogenesis if it allows further seizure spread through the entorhinal cortex–dentate gyrus–hippocampus–entorhinal cortex circuit. It could also contribute to the excitotoxic pattern of principal cell loss in the hippocampus proper, which is a hallmark of chronic temporal lobe epilepsy. In addition, because heterosynaptic depression is recruited by synchronous mossy fiber discharges, it could contribute to seizure termination; a breakdown in this phenomenon could thus lead to the persistence of seizure activity during status epilepticus. Others have noted a downregulation of $GABA_B$ receptors on presynaptic terminals of inhibitory cells in the dentate gyrus in animals 2 weeks after kainic acid-induced seizures, leading to reduced disinhibition and an increase in GABA release (Haas et al., 1996). Although we were unable to detect a significant loss in autoradiographic labeling in the molecular layer of the dentate gyrus, such an effect could reflect a compensatory antiepileptic effect in the chronic stages of epilepsy. Complex changes in $GABA_B$ receptor expression have also been reported in the entorhinal cortex during epileptogenesis, including presumed loss of postsynaptic $GABA_B$ receptors (Kokaia and Kokaia, 2001) and an increase in presynaptic $GABA_B$ receptor-mediated autoinhibition in layer III of the entorhinal cortex (Gloveli et al., 2003). These changes are likely to have a proepileptic effect, enhancing the entorhinal cortex to hippocampus signaling. Thus, changes in $GABA_B$ receptor expression at multiple sites in the hippocampal formation during epileptogenesis may have complex effects on excitability.

In summary, we have shown that $GABA_B$ receptor-mediated heterosynaptic depression at the mossy fiber synapse is reduced acutely after SE, despite an increase in the GABA concentration in mossy fibers. This is associated with a decrease in the sensitivity of the mossy fiber response to the $GABA_A$ agonist baclofen, a change that persists for at least 3 weeks. This can be explained by a loss of presynaptic $GABA_B$ receptors on mossy fiber terminals, revealed by autoradiographic detection of bound $GABA_B$ receptor radioligand in the stratum lucidum. Reduced $GABA_B$ receptor-mediated heterosynaptic depression after SE may play a role in the maintenance of self-sustaining seizure activity and the later development of spontaneous seizures.

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